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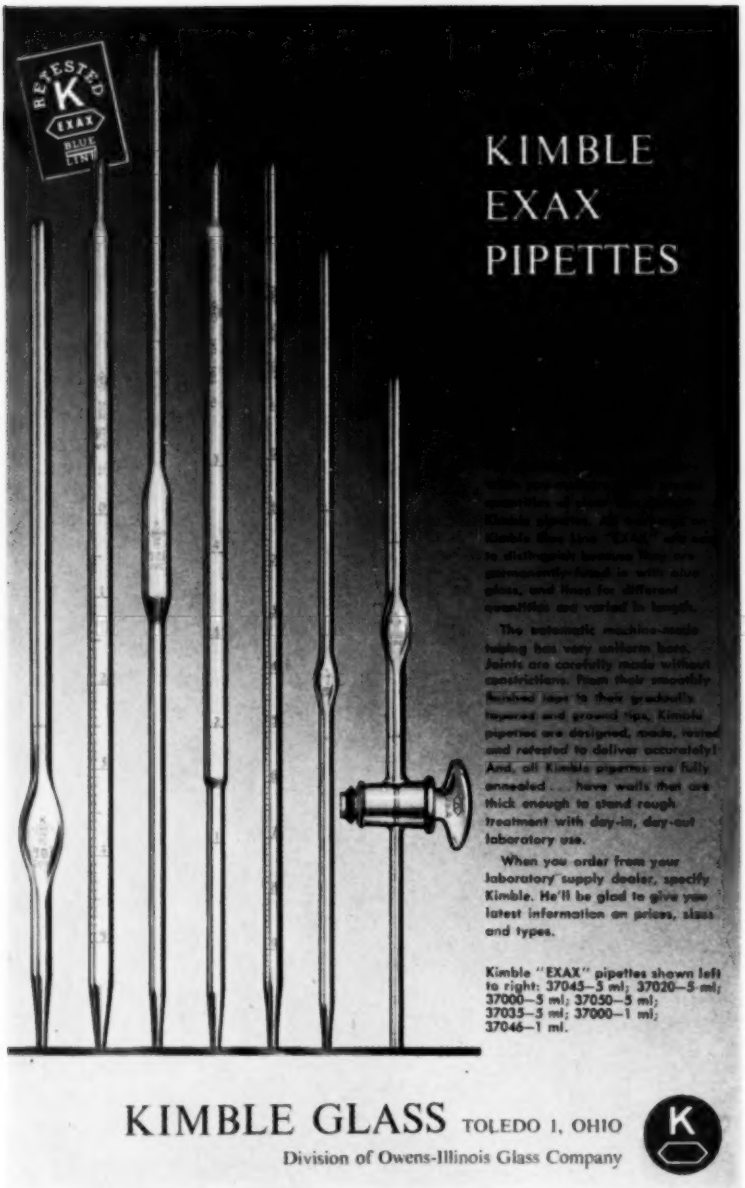

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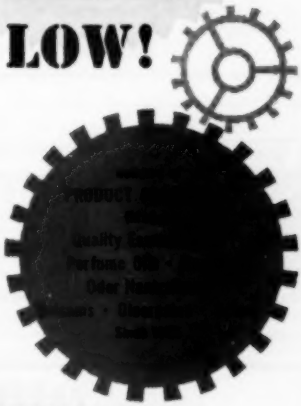

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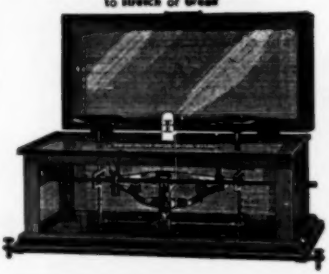

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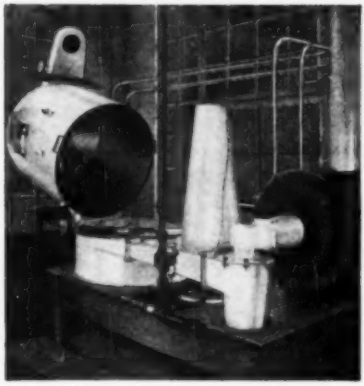

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Rapid Colorimetric Determination of Formaldehyde in Toxoids*

By MORRIS B. JACOBS, ELEANOR L. EASTMAN, and DAVID L. SHEPARD

The phenylhydrazine-ferricyanide colorimetric formaldehyde method has been modified for use in the estimation of residual formaldehyde in detoxified bacterial filtrates. By adding the reagents in a fixed order, the magenta color produced in this reaction has been stabilized. This permits colorimetric determinations with the aid of a photoelectric colorimeter and the use of a standard curve. The method has accuracy and precision and is capable of being used for the estimation of 1 part of formaldehyde in 500,000 parts of original sample solution.

THE National Institutes of Health (8, 9) has set certain requirements for the amount of "residual" formaldehyde permitted in fluid toxoids, namely, "The permissible residual must not exceed 0.02 per cent of free formaldehyde, CH_2O, or 0.05 per cent of solution of formaldehyde containing 37.0 per cent of formaldehyde."

One of the methods used for the determination of residual formaldehyde in toxoids is a gravimetric method in which the formaldehyde is precipitated by use of dimethyldihydroresorcinol in acetic acid solution (13, 14). Another method (2) used for this purpose is a variation of the fuchsin-sulfite or rosaniline-sulfite method (3, 15).

The gravimetric method is tedious, time-consuming, and requires substantial quantities of toxoid if accuracy and precision are to be obtained. The fuchsin-sulfite method has the marked disadvantage that the color developed increases on standing so that a set of standards must be run for each set of determinations and an arbitrary reading time must be selected. It is difficult to adapt this method for photoelectric colorimetric estimation.

We have found that a modification of the phenylhydrazine-ferricyanide method, sometimes termed the Schryver method, can be used for the rapid and accurate estimation of formaldehyde in toxoids. A standard curve can be made for such determinations with a given photoelectric instrument so that it is not necessary to prepare new standards for each set of determinations.

Matsukawa (5) also used a variation of the phenylhydrazine-ferricyanide method. In this variation, he used 2 ml. of test solution containing from 0.02 to 1.0 mg. per cent of formaldehyde. He employed an 8 per cent solution of sodium chloride, a 0.5 per cent solution of phenylhydrazine hydrochloride in 50 per cent ethyl alcohol, and heating at 40° for five to ten minutes for the reaction between the phenylhydrazine hydrochloride and the formaldehyde. He prepared a solution of potassium ferricyanide by dissolving 2.5 Gm. in 5 ml. of water and then in 40 ml. of absolute alcohol, which solution he subsequently diluted with additional water to 100 ml. He

* Received March 22, 1951, from the Bureau of Laboratories, Department of Health, City of New York.

added 0.5 ml. of this ferricyanide reagent and then 0.75 ml. of hydrochloric acid (1:1) to the test mixture and stirred thoroughly. The mixture was then kept in a water bath for ten minutes at 15°. After the development of the color, it was read in a Pulfrich photometer with an S-53 type filter.

Subsequent work by Matsukawa (6) lead him to abandon this method and to adopt an oxidimetric method involving a prior precipitation of the formaldehyde with dimethyldihydroresorcinol and subsequent oxidation of the isolated aldomedone with dichromate. Both methods of Matsukawa are far more complex than the method presented here.

Among the modifications of the phenylhydrazine-ferricyanide method presently used for the determination of formaldehyde in air and water are those of Kersey, Maddocks, and Johnson (4); Barnes and Speicher (1); and Moskowitz, Siegel, and Burke (7). These have been reviewed by Jacobs (3).

By altering the preparation of the reagents and by changing the order of the addition of these reagents in combination with changes in technique, we have been able to stabilize the color produced in this reaction so that photoelectric measurements can be made.

METHOD

Reagents

Potassium Ferricyanide Solution.—Prepare a 5% solution of potassium ferricyanide by dissolving 5 Gm. of $K_3Fe(CN)_6$, analytical reagent, in distilled water and make up to 100 ml. of solution in a volumetric flask. This solution must be prepared fresh daily.

Phenylhydrazine Reagent.—Dissolve 1.38 Gm. of phenylhydrazine hydrochloride, $C_6H_5NHNH_2$.HCl, in distilled water and make up to 100 ml. of solution in a volumetric flask. This solution must be prepared daily.

Standard Formaldehyde Solution.—Transfer 8.2 ml. of 37% formalin solution to a liter volumetric flask and dilute to 1 L. with distilled water. This solution contains approximately 3 mg./ml. The exact concentration was estimated by the Ripper bisulfite method (10, 12). The standard potassium sulfite solution was prepared from potassium metabisulfite which was standardized against standard iodine solution, standardized in turn against a primary potassium dichromate standard solution.

Procedure

Transfer 2 ml. of each of the toxoid samples with the aid of pipettes to 10-ml. volumetric flasks and make to volume with distilled water. Mix thoroughly.

Set up a series of half-ounce or 15-ml. glass-stoppered vials, test tubes, or bottles. Transfer a 1- or 2-ml. aliquot of the diluted sample to each of these glass-stoppered vessels. Add 4 or 3 ml. of distilled water to bring the volume to 5 ml. Mix. Add 1 ml. of 5% potassium ferricyanide solution, restopper, and shake. Add 4 ml. of concentrated hydrochloric acid with the aid of a burette or with a pipette equipped with a safety pipetter (11), restopper and shake. Add 2 ml. of 1.38% phenylhydrazine hydrochloride solution, and shake thoroughly. Allow to stand for fifteen minutes. Transfer the solution to the colorimeter tube and back to the reaction vessel several times to free the solution of all gas bubbles. Finally transfer to the colorimeter tube and read the magenta color produced. We used a Klett-Summerson colorimeter with the No. 54 green filter.

Procedure for Standard Curve

In order to make allowance for the coloring matter normally present in the media used for the production of the toxoid and in the crude bacterial filtrate, some crude bacterial toxin solution before conversion to toxoid was added to known formaldehyde concentrations.

TABLE I.—COMPARISON OF COLORIMETRIC AND GRAVIMETRIC METHODS ON TOXOID SAMPLES OF UNKNOWN FORMALDEHYDE CONCENTRATION

Toxoid	Number	Colorimetric Determination, %			Gravimetric Determination, %		
		[a]	[b]	Av.	[c]	[c]	Av.
Diphtheria	94	0.013	0.013	0.013	0.013	...	...
Diphtheria	95	0.011	0.013	0.012	0.016	0.014	0.015
Diphtheria	96	0.014	0.014	0.014	0.014	0.017	0.016
Diphtheria "P" fraction	MS40	0.048	0.048	0.048	0.041	0.039	0.040
Diphtheria "P" fraction	1291	0.039	0.036	0.038	0.035	0.029	0.032
Diphtheria	I	0.025	0.024[d]	0.025	0.029	0.028	0.029
Diphtheria	II	0.023	0.024[d]	0.024	0.027	0.027	0.027
Diphtheria	III	0.026	0.025[d]	0.026	0.027	...	...
Diphtheria	IV	0.029	0.026[d]	0.028	0.029	0.025	0.027
Diphtheria	V	0.027	0.026[d]	0.027	0.027	0.029	0.028
Tetanus	62	0.014	0.016	0.015	0.015	0.017	0.016
Tetanus	63	0.020	0.018	0.019	0.0195[e]	...	...
Tetanus	65	0.020	...	...	0.019[e]	0.021[e]	0.020
Tetanus	66	0.0148	0.0140	0.014	0.016	0.014	0.015

[a] 1/5 ml. sample; [b] 2/5 ml. sample; [c] 5 ml. sample; [d] 1/10 ml. sample; [e] 10 ml. sample.

Dilute 1 ml. of crude bacterial toxin solution to 10 ml. with distilled water in a volumetric flask. Transfer 2 ml. of this diluted toxin solution to a series of 15-ml. glass-stoppered bottles. Add 1, 2, or 3 ml. of formaldehyde solution containing known concentrations of formaldehyde so that a series of formaldehyde concentrations at 10 to 15 μg. intervals covering the range from 10 to 120 μg. is obtained.

RESULTS

Figure 1 shows the standard curve obtained by this method using known quantities of formaldehyde solution. The results of two analysts working independently are plotted.

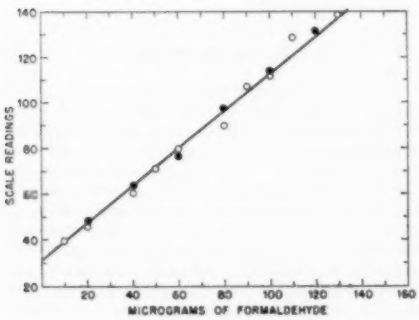

Figure 1

In Table I the results obtained by use of this colorimetric method and with the dimethyldihydroresorcinol gravimetric method are tabulated. These samples were routine samples.

Five separate standard curves were prepared originally. Some were prepared using water as the zero reading and others were prepared using a blank as the zero reading. Since virtually all had the same slope, it was decided to use water for the adjustment to zero reading of the colorimeter.

DISCUSSION

It is customary to add the phenylhydrazine reagent before the addition of the ferricyanide reagent (1, 4, 5, 7). We found that while this procedure gives a more intense magenta color, this color is markedly unstable, and furthermore a turbidity often results. The production of a turbidity is almost unavoidable if the phenylhydrazine reagent is added before the addition of the hydrochloric acid. When the acid and phenylhydrazine are mixed together just prior to addition to the test solution, the formation of turbidity is minimized, but the stability of the reaction color is not promoted. By adding the phenylhydrazine hydrochloride reagent last, the color produced is less intense but we achieve stability. The reduction in the sensitivity of the estimation is not too great. Thus the intensity is great enough to enable us to determine 10 μg. of formaldehyde in 12 ml. of total test solution or a sensitivity of 1 part in 1 million. This sensitivity is equivalent to at least 1 part in 100,000 of the original sample. This order of sensitivity is entirely adequate for the analysis of formaldehyde in toxoids since the requirements of the National Institutes of Health permit up to 200 μg./ml.

It is necessary to take care, prior to reading, that all bubbles are eliminated by transferring the test solution from tube to vessel by pouring down the side of the tube before reading.

It can be seen from the standard curve that we obtained greater precision in the range from 20 to 100 μg. than for concentrations above this range. Hence it is preferable to dilute or adjust the test sample so that an aliquot falling within this range can be withdrawn for the test. We found that the precision obtained with the colorimetric phenylhydrazine-ferricyanide method was superior to that obtained with the methone gravimetric method, particularly for the lower concentrations. This was also noted by Matsukawa.

When a new bottle of reagent is used, it is probably good practice to recheck the blank reading and the standard curve. It is to be noted that the standard curve shown in Fig. 1 was prepared from the results obtained by two individuals.

The sensitivity of the reaction can be increased by extracting the color produced with a solvent such as isoamyl alcohol or isobutyl alcohol. The solvent extract can then be placed in a microcolorimeter tube and the intensity of the color can be measured.

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The Separation of Some of the Cinchona Alkaloids by Paper Partition Chromatography*,†

By DONALD J. LUSSMAN, ERNST R. KIRCH, and GEORGE L. WEBSTER

Paper chromatograms have been prepared using cyclohexanol or cyclohexanone and dilute hydrochloric acid as the solvent. Identification of the position of individual cinchona alkaloids upon the chromatogram was accomplished by means of R_F values determined with pure samples, coupled with the observation of fluorescence in the cases of quinine and quinidine and the development of a nonfluorescent colored compound by cinchonidine and cinchonine when the chromatogram was treated with a potassium iodoplatinate reagent. As little as 3 μg. of quinine or quinidine, 25 μg. of cinchonidine, and 100 μg. of cinchonine may be detected by the method.

PRESENT commercial methods of separation of the principal alkaloids of cinchona employ various modifications of continuous extraction procedures, using ammoniacal alcohol as the extracting menstruum (1, 2). Chromatographic methods have also been presented to accomplish this separation, as described by Heilbron (3). The individual alkaloids may then be separated by the preparation of various salts of the alkaloids whose solubilities vary sufficiently to allow separation in a relatively pure state. Partial separations may also be made by fractional crystallizations from ammoniacal alcohol at low temperatures.

Since the efficiency of existing methods of separation of the cinchona alkaloids is somewhat questionable, commercial preparations of these alkaloids are known at times to contain appreciable amounts of other alkaloids as impurities. Doubtless this is unavoidable; it does not constitute a serious situation, however, unless the content of these impurities reaches an amount sufficient to produce a change in the pharmacological action of the preparation. Since such impurities frequently cannot be avoided, it is desirable to have available an analytical procedure whereby these impurities may be detected, even when present in minute amounts.

The purpose of this investigation was to develop such a procedure. Since the introduction of paper partition chromatography methods by Consden, Gordon, and Martin (4), a method has been available for the identification of materials present in very small amounts. For this reason, we felt that paper partition chromatography might be applicable for the above analytical procedure.

* Received April 2, 1951, from the Department of Chemistry, University of Illinois, College of Pharmacy, Chicago.

† An abstract of a thesis submitted to the Graduate College of the University of Illinois by Donald J. Lussman in partial fulfillment of the requirements for the degree of Master of Science in Pharmaceutical Chemistry.

EXPERIMENTAL

The apparatus used in this determination was similar to that described by Williams and Kirby (5), as modified by Wolfson, Cohn, and Devaney (6). The chamber consisted of a glass jar, 24 in. high and 6 in. in diameter. The jar was used in an upright position, the solvent being placed in the bottom of the jar itself. A glass plate of suitable size was used as the closure for the top of the jar, and a suitable lubricant was used to make an airtight seal. The paper, in the form of a cylinder, was then placed in an upright position in the jar, so that the lower edge of the paper was immersed in the solvent. Spotting of stock solutions onto the paper was accomplished by the use of calibrated capillary pipettes, from which a measured amount of stock solution was allowed to flow onto the paper. The paper was then allowed to dry by evaporation of the solvent.

Similar determinations using a descending-type apparatus similar to that described by Consden, Gordon, and Martin (4) were also carried out with equally successful results.

Of a number of solvents investigated, including phenol, collidine, pyridine, butanol-acetic acid, benzyl alcohol, and others, the most efficient separations were obtained using cyclohexanol. The latter was used in conjunction with the vapors from various concentrations of hydrochloric acid saturating the chamber. The most efficient of these separations was accomplished by the use of hydrochloric acid in dilutions ranging from 1:3 to 1:6 (about 4.0 to 2.0 *N*).

The determinations were carried out under two different sets of conditions. In the first of these, the cyclohexanol was saturated with water, and the dilute acid placed in a separate container within the chamber, so that the vapors saturated the chamber during development of the chromatogram. Under the second set of conditions, the solvent was saturated with the water-acid mixture instead of with distilled water alone. This was carried out by mixing the cyclohexanol, water, and acid in a separatory funnel. The cyclohexanol layer was then removed and placed in the bottom of the chamber, and the aqueous layer placed in a separate container inside the chamber. The solutions were prepared from equal volumes of organic solvent and acid-water mixture. The method of mixing was the same as that described by Foster, McDonald, and Jones (7)

in their separations of the water-soluble ergot alkaloids. Both of the above solvent mixtures produce a satisfactory separation of the alkaloids, although rather wide variations in the R_F values[1] did exist. This difficulty was readily overcome by running a series of known samples of recrystallized alkaloids[2] simultaneously with the unknown so that the bands can be identified positively by comparison.

Following development of the chromatogram itself, identification of the separated alkaloids was accomplished by two different methods, depending upon the alkaloid. In the case of quinine and quinidine, identification was relatively simple, since these materials fluoresce when exposed to ultraviolet light. However, since cinchonine and cinchonidine are not fluorescent, a color reagent had to be used for their identification. The most suitable of these was found to be the potassium iodoplatinate reagent[3] described by Zaffaroni, Burton, and Keutmann (8) for the identification of ketosteroids. Dilution of this reagent with four parts of distilled water provides a suitable reagent for the identification of all four of the major alkaloids of cinchona.

Results obtained by the above methods, as shown in Tables I, II, and III, represent the average of four or more determinations.

A similar series of chromatograms was developed using cyclohexanone as the solvent; separations in these determinations were not as good as those obtained using cyclohexanol. This was especially true when the water-saturated solvent was used instead of the solvent saturated with dilute acid. In the former case, separations were very erratic and could not be reproduced consistently. In the latter case, suitable separations could be made, especially when separating quinine from quinidine. The approximate RF values obtained by the use of cyclohexanone as solvent are shown in Table III.

The filter papers used in these determinations were Whatman No. 1 and Schleicher and Schuell No. 576. No appreciable differences in RF values were noted between the various samples of paper.

DISCUSSION

The most efficient separations apparently were those obtained by using cyclohexanol saturated with distilled water; experience has shown, however, that although a greater variation in RF values was noted between the various alkaloids when using this method, more reproducible results were obtained when the cyclohexanol was saturated with dilute acid. The reason for these variations is not known.

The single advantage of using cyclohexanone rather than cyclohexanol as a solvent is in the rate of development of the chromatogram. It was found that the average chromatogram required thirty-six to forty-eight hours to develop using cyclohexanol as the solvent, as compared to six to eight hours when cyclohexanone was used. However, the efficiency of the separations is much in favor of cyclohexanol, since the bands obtained by using cyclohexanone show a tendency to spread and distort easily.

Determinations were carried out using both the free alkaloids and the alkaloid salts. No appreciable difference in the results was noted for quinine and quinidine, whereas slightly lower RF values were noted for cinchonine and cinchonidine alkaloids than were noted for the corresponding salts.

The sensitivity of the separations varied widely with the alkaloids under study. In the cases of quinine and quinidine which were identified by their fluorescence, quite small quantities, as little as 3 μg., could be identified by exposure of the developed chromatogram to ultraviolet light. Since cinchonine and cinchonidine are not fluorescent, it was necessary to identify them by means of the color reaction described above. Test tube experiments involving use of the reaction showed that the levorotatory

[1] R_F is the ratio of the distance traveled by the band to the distance traveled by the solvent, measured from the point at which the substances under test were applied to the paper.

[2] All alkaloids used were of U. S. P. or N. F. grade, twice purified by dissolving in dilute hydrochloric acid and extracting with ether after neutralization with sodium hydroxide.

[3] The reagent is prepared as follows: "5 ml. of 5 per cent platinum chloride in 1 *N* hydrochloric acid, 45 ml. of 10 per cent potassium iodide and 100 ml. of distilled water were combined, and the solution stored in a dark, glass-stoppered bottle."

TABLE I.—RF VALUES OF CINCHONA ALKALOIDS USING CYCLOHEXANOL SATURATED WITH WATER, AND DILUTE ACID IN SEPARATE CONTAINER INSIDE CHAMBER

HCl Concentration	Quinine	Quinidine	Cinchonine	Cinchonidine
1:5 (approx. 2.5 *N*)	0.25 ± 0.05	0.75 ± 0.05	0.20 ± 0.05	0.32 ± 0.05
3:10 (approx. 3.5 *N*)	0.34 ± 0.05	0.55 ± 0.05	0.27 ± 0.05	0.48 ± 0.05

TABLE II.—RF VALUES OF CINCHONA ALKALOIDS USING CYCLOHEXANOL SATURATED WITH DILUTE ACID, WITH EXCESS DILUTE ACID IN SEPARATE CONTAINER INSIDE CHAMBER

HCl Concentration	Quinine	Quinidine	Cinchonine	Cinchonidine
1:5 (approx. 2.5 *N*)	0.45 ± 0.03	0.51 ± 0.03	0.35 ± 0.05	0.27 ± 0.05
3:10 (approx. 3.5 *N*)	0.51 ± 0.03	0.58 ± 0.03	0.42 ± 0.05	0.34 ± 0.05

TABLE III.—RF VALUES OF CINCHONA ALKALOIDS USING CYCLOHEXANONE SATURATED WITH DILUTE ACID, WITH EXCESS DILUTE ACID IN SEPARATE CONTAINER INSIDE CHAMBER

HCl Concentration	Quinine	Quinidine	Cinchonine	Cinchonidine
1:5 (approx. 2.5 *N*)	0.32 ± 0.05	0.43 ± 0.05	0.09 ± 0.03	0.06 ± 0.03
3:10 (approx. 3.5 *N*)	0.21 ± 0.04	0.31 ± 0.04	0.03 ± 0.03	0.05 ± 0.03

alkaloids, quinine and cinchonidine, are more sensitive to the reaction than are the dextrorotatory alkaloids, quinidine and cinchonine. For this reason it was much easier to identify cinchonidine than it was to identify cinchonine. In general, approximately 25 μg. of cinchonidine were easily identifiable by this reaction, whereas approximately 100 μg. of cinchonine were required before identification was possible.

A series of determinations was also carried out on totaquine, U. S. P., in an attempt to test the efficiency of the method of analysis. Quinine was identified readily by its characteristic fluorescence, and cinchonidine was easily located by application of the color reaction. Only in cases where large amounts of totaquine were used was the cinchonine detected with any degree of certainty. Quinidine was not detected in any of the determinations.

SUMMARY

A method has been described for the separation of the major alkaloids of cinchona by means of paper partition chromatography, using cyclohexanol or cyclohexanone with hydrochloric acid as the solvent mixture. The separated bands were then identified either by their fluorescence or by the application of a color reaction using potassium iodoplatinate reagent.

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The Colorimetric Determination of the Antihistaminic Drug N^1-*p*-Methoxy Benzyl-N^1-2-Pyridyl-N^1-Dimethyl Ethylene Diamine (Neohetramine)*

By MILTON FELDSTEIN and NIELS C. KLENDSHOJ

A colorimetric method for the determination of Neohetramine in biological material and pharmaceutical preparations is described. Extracted residues containing Neohetramine are heated with 2-thiobarbituric acid, and the resulting color is measured in a spectrophotometer at 510 mμ.

THE REACTION between 2-thiobarbituric acid and 2-sulfanilamido pyrimidine to form a red color was reported by Kohn and Læversedge (1) in 1942. Further investigation of this colorimetric reaction was described by Shepherd (2), who showed that the reaction is specific for the pyrimidine group, with substituents in the 2-position, and with the 4-, 5-, and 6-position unsubstituted. Among the antihistaminics in vogue at present, N^1-*p*-methoxy benzyl-N^1-2-pyridyl-N^1-dimethyl ethylene diamine (Neohetramine) appears to be the only one containing the pyrimidine nucleus (3). The present study was undertaken to adapt the reaction to the determination of Neohetramine in blood, urine, tissue extracts, and tablets. Optical measurements of the colored reaction product were made with the Beckman Model DU Spectrophotometer, using 1-cm. absorption cells.

When the procedure described by Shepherd (2) was followed, it was found that only large amounts of Neohetramine gave a colored reaction product. As a result, it became necessary to modify the procedure and determine the optimum conditions for the development of color with amounts of Neohetramine which would normally be encountered in body fluids.

* Received January 25, 1951, from the Division of Toxicology, University of Buffalo, School of Medicine, and the Department of Biochemistry, Buffalo General Hospital.

PROCEDURE

Urine.—The *p*H of 50 ml. of urine is adjusted to 7.8 by the addition of 2.0 Gm. of anhydrous Na_2HPO_4. After solution of the salt, the urine is extracted with 100 ml. of carbon tetrachloride in a separatory funnel. After separation of the layers, the solvent is washed with 50 ml. of water, filtered into a second separatory funnel, and extracted with 10 ml. of 0.1 *N* hydrochloric acid. The HCl layer, which contains the Neohetramine, is centrifuged to remove small droplets of solvent and saved for reaction with 2-thiobarbituric acid.

Blood.—Five milliliters of oxalated blood is placed in a separatory funnel. Ten milliliters of water and 2 ml. of 28% ammonia are added. The funnel is rotated to hemolyze the blood, and the mixture is extracted with 100 ml. of carbon tetrachloride. The solvent is treated as indicated above for urine.

Tissue.—Fifty grams of tissue is placed in a Waring blender, and 2 ml. of concentrated ammonia and 200 ml. of ethylene chloride are added. (Ethylene chloride was found to have less tendency to form emulsions with tissue.) The mixture is homogenized for one to two minutes and allowed to stand until the layers separate. The ethylene chloride layer is filtered and a 100-ml. aliquot is extracted with hydrochloric acid as described above for urine.

Tablets.—Tablets are powdered in a mortar and are quantitatively transferred to a 500-ml. volumetric flask with the aid of 0.1 *N* HCl. The solution is diluted to the 500-ml. mark with 0.1 *N* HCl and mixed. After filtration, an aliquot is removed and treated as indicated in the procedure for urine.

Five-milliliter aliquots of the hydrochloric acid extracts are placed in 100-ml. beakers. Two milliliters of a saturated solution of 2-thiobarbituric acid in 1 *N* hydrochloric acid is added and the beakers placed on a hot plate which is regulated at a temperature of 110–130°. The solutions are allowed to evaporate to dryness, and are heated for an additional two hours. After cooling, 10 ml. of 2.8% ammonia is added, and the contents of the beaker are stirred with a glass rod to insure complete solution of the residues. The optical densities are measured within ten minutes in the Beckman spectrophotometer at 510 mμ with distilled water as a blank. The 10-ml. volume is satisfactory for amounts of Neohetramine between 0.01 and 0.06 mg. When larger amounts are present, the final volume is made to 100 ml.

Determination of Optimum Wave Length.—Aliquots of Neohetramine solution containing 0.01 to 0.1 mg. were treated as indicated in the procedure, and the optical density measured at various wave lengths. Figure 1 shows the density-wave length curve of the alkalinized color. Inspection of the curve indicates that the optimum wave length is 510 mμ.

Determination of Stability of Color.—Colored solutions were prepared as indicated above, and the densities at 510 mμ read at various time intervals. It was found that the color is stable for fifteen minutes, after which it begins to fade.

Adherence to Beer's Law.—Varying amounts of Neohetramine were treated as described in the procedure, and optical densities at 510 mμ measured. Figure 2 shows the concentration-density curves.

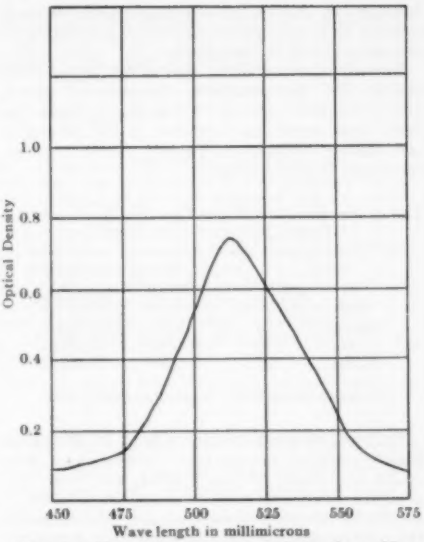

Fig. 1.—Absorption spectrum of dye from Neohetramine and thiobarbituric acid in ammoniacal solution. (0.05 mg./10 ml.)

For amounts of Neohetramine between 0.01 and 0.06 mg., the final volume selected was 10 ml. For amounts between 0.1 and 0.5 mg. the final volume

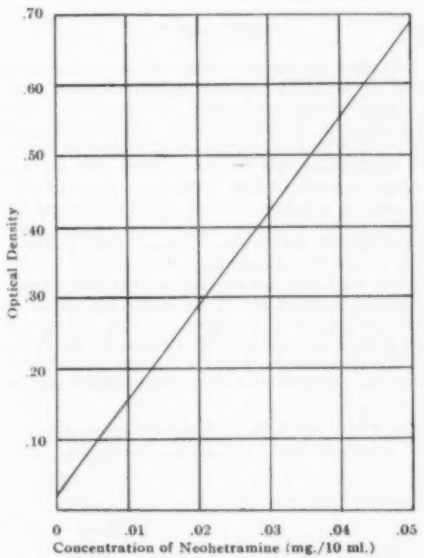

Fig. 2.—Concentration-density curve for the reaction product between Neohetramine and thiobarbituric acid.

10 ml. volume, wave length 510 mμ, Beckman Spectrophotometer.

selected was 100 ml. The straight lines obtained indicate that the optical density is a function of concentration of Neohetramine.

Determination of Blank Values for Tissue Containing No Neohetramine.—Samples of blood, urine, and brain known to contain no Neohetramine were treated as indicated in the procedure and optical densities measured. Table I shows the results of these analyses.

TABLE I.—OPTICAL DENSITIES OF EXTRACTS OF NORMAL BIOLOGICAL MATERIALS

Tissue	No. of Determinations	Av. Optical Density of Reaction Product	Correction Mg. Neohetramine (10 Ml. Vol.)
Blood	25	0.036[a]	0.0025
Urine	25	0.036[a]	0.0025
Brain	16	0.036[a]	0.0025

[a] Average deviation, 0.005; standard deviation, 0.007.

Recovery of Neohetramine Added to Biological Fluids.—Various amounts of Neohetramine were added to samples of blood, urine, and brain and analyzed according to the procedure. Table II shows the results of these analyses. Inspection of the colored residues after reaction with 2-thiobarbituric acid will determine the final volume (10 ml. or 100 ml.) necessary. For amounts of Neohetramine above 0.5 mg., smaller aliquots are analyzed to bring the concentration within the desired range.

Analysis of Tablets.—Table III shows the results of analyses of Neohetramine tablets from various sources.

TABLE II.—RECOVERY OF NEOHETRAMINE ADDED TO BIOLOGICAL MATERIALS

Tissue	No. of Determinations	Quantity of Neohetramine Added, Mg.	Average Recovery, Mg.
Blood,	10	0.020	0.019 ± 0.002[a]
5-ml.	10	0.050	0.048 ± 0.002[a]
samples	10	0.090	0.092 ± 0.002[a]
Urine,	10	0.020	0.019 ± 0.002[a]
50-ml.	10	0.050	0.048 ± 0.002[a]
samples	10	0.090	0.092 ± 0.002[a]
Brain,	5	0.050	0.052 ± 0.002[a]
50-Gm.	5	0.100	0.104 ± 0.003[a]
samples	5	0.300	0.296 ± 0.003[a]

[a] Average deviation.

TABLE III.—ANALYSIS OF NEOHETRAMINE TABLETS

Tablet No.	Analysis on Label, Mg. Neohetramine	Found, Mg. Neohetramine
1	50	48.7 ± 0.2[a]
2	50	46.4 ± 0.2[a]
3	50	49.1 ± 0.3[a]
4	50	51.3 ± 0.2[a]
5	50	48.9 ± 0.2[a]

[a] Average deviation.

DISCUSSION

Analysis of normal tissues (Table I) indicates that the naturally occurring pyrimidine compounds present in biological material do not interfere with the reaction. Sulfadiazine and sulfamerazine, both of which contain the pyrimidine nucleus and both of which react with 2-thiobarbituric acid, are not extracted from alkaline solution by carbon tetrachloride or ethylene chloride, and thus present no interference. Thiamine, which is a 2-methyl pyrimidine, also has substituents in the 5- and 6-position, and hence does not react with the reagent.

The minimum amount of Neohetramine which can be determined is 0.01 mg. in a 5-ml. aliquot of the hydrochloric acid extract. Larger volumes of the acid extract may be taken for analysis, but doing so would introduce larger correction factors and thus decrease the accuracy of the determination. A value of 0.01 mg. in 5 ml. of acid extract corresponds to 0.40 mg. % Neohetramine for blood, 0.04 mg. % for urine, and 0.08 mg. % for brain when the procedures described previously are followed. When greater concentrations of the drug are expected, smaller aliquots or smaller samples of tissue may be taken for analysis.

SUMMARY

A colorimetric method for the determination of Neohetramine in biological material and pharmaceutical preparations is described. Extracted residues containing Neohetramine are heated with 2-thiobarbituric acid, and the resulting color is measured in a spectrophotometer at 510 mμ.

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1,2,3,4-Tetrahydrocarbazolylcarboxylic Acid Esters with Local Anesthetic Activity*

By HUBERT W. MURPHY

The local anesthetic activity, toxicity, and irritation displayed by the hydrochlorides of β-diethylaminoethyl, 3-(2-methylpiperidino)propyl, 3-di-*n*-butylaminopropyl, 3-morpholinopropyl, and 1-methyl-3-diethylaminopropyl esters of 1,2,3,4-tetrahydro-6-carbazolylcarboxylic acid have been studied. The local anesthetic activity of the ester-hydrochlorides of the isomeric acid with carboxyl linked in position 8 has been found to be lacking or of a very low order. However, ester-salts of either acid were capable of effecting smooth muscle relaxation. The germicidal activities of some of the ester-hydrochlorides have been compared with phenol by the standard F. D. A. test. The 3-di-*n*-butylaminopropyl ester salts appear to be the best local anesthetics of the compounds prepared; however, the irritation inherent in all members of the series would appear to limit their wide application as local anesthetics.

THE HYDROCHLORIDE of β-diethylaminoethyl 1,2,3,4-tetrahydro-6-carbazolylcarboxylate prepared in the course of a previous investigation (1)[1] was discovered to display a marked local anesthetic activity when applied topically. It therefore seemed desirable to extend the study of the local anesthetic activity of dialkylaminoalkyl esters of the carbazole acids with a partially hydrogenated nucleus. All these esters as their hydrochlorides, except the 3-morpholinopropyl, of 1,2,3,4-tetrahydro-6-carbazolylcarboxylic acid displayed a potency and toxicity comparable to dibucaine, but were much more stable in aqueous solution. All solutions displayed a slight fluorescence.

Quite unexpectedly and in contrast to Burtner's esters (2), wherein the carboxyl group was linked at various positions on the symmetrical carbazole nucleus to furnish active local anesthetics, we found the β-diethylaminoethyl (1) and 3-(2-methylpiperidino)propyl esters of 1,2,3,4-tetrahydro-8-carbazolylcarboxylic acid to be completely lacking in local anesthetic activity when applied in concentrations as high as 2 per cent.

Esters of the tetrahydrocarbazole with the carboxyl group linked with either the 6- or 8-position produced distinct neurotropic inhibition of the activity of isolated rabbits' intestines suspended in Tyrode's solution in concentrations ranging from 1:200,000 to 1:500,000.

The hydrochlorides of all (except the 3-morpholinopropyl) 1,2,3,4-tetrahydro-6-carbazolylcarboxylates were difficultly soluble in water and required heating to expedite the preparation of solutions. The solutions prepared in this manner, however, appeared to be quite stable. These ester-salts are also substantially insoluble in physiological saline solution. The sulfates were much more soluble, but in concentrations greater than 0.25 per cent they were observed to precipitate in rabbits' eyes.

When solutions of the ester-salts in concentrations greater than 0.1 per cent were applied to rabbits' corneas, deep anesthesia was induced accompanied by irritation, the extent of the latter depending upon the concentration. Concentrations of 0.5 per cent in each instance produced profound anesthesia with a duration of greater than four hours, and severe irritation of the cornea and conjunctiva with intense secretion.

Concentrations of 0.1 per cent of the salts, in most instances, proved to be the most desirable since these induced effective anesthesia with negligible or only slight irritation.[2]

In order to obtain statistical significance, several lots of each ester-salt were prepared by at least two different methods of esterification and from different lots of raw materials. The ester-salts were subjected to pharmacological investigation simultaneously and, later, individually. No significant differences were observed with various purified lots of the same compound. The experimental data reproduced in Tables I and II are averaged.

* Received January 26, 1951, from the Pharmaceutical Research Department, Eli Lilly Research Laboratories, Indianapolis, Ind.

[1] After these compounds had been prepared and studied it was discovered that Burtner had previously reported the preparation of the hydrochloride of diethylaminoethyl ester of 1,2,3,4-tetrahydro-6-carbazolylcarboxylic acid [*J. Am. Chem. Soc.*, **62**, 528(1940)] which he designated as the ester of 6,7,8,9-tetrahydro-carbazole-2-carboxylic acid. This compound has been correctly recorded in *Chem. Abstr.* [**34**, 2841 (1940)], but is incorrectly indexed as the ester hydrochloride of 6,7,8,9-tetrahydro-3-carbazolecarboxylic acid [**34**, 8042 (1940)].

[2] Irritation: (*a*) negligible—difficult to differentiate the site of injection from adjacent normal tissue; (*b*) slight—a trace of hyperemia or ischemia or a point of elevation with no gross pathology; (*c*) mild—hyperemia or ischemia with some fibrosis; (*d*) moderate—hyperemia or ischemia extending to an area of more than 1 cm.², fibrosis with extension, and edema.

Some of these ester-salts, as well as other known local anesthetics, have been examined for bacteriostatic activity. Our data indicate that bacteriostatic activity is displayed in varying degrees by all the local anesthetics studied and, moreover, that among the topical anesthetics the effective local anesthetic concentration corresponds approximately to that required to kill or inhibit *Staphylococcus aureus* at 37° in ten minutes. Although this desirable bactericidal or bacteriostatic activity is displayed, it must be emphasized that it does not indicate that solutions are self-sterilizing because spores are apparently resistant.

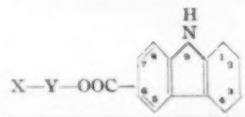
The approximate phenol coefficients as determined by the Food and Drug Administration germicidal test are reported in Table III.

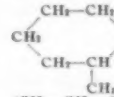
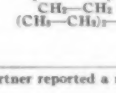
EXPERIMENTAL

The preparation of 1,2,3,4-tetrahydro-6-carbazolylcarboxylic acid and the 8-isomer has been described (1, 3).

The process found most satisfactory for the preparation of dialkylaminoalkyl or heterocycloalky

TABLE I.—ESTERS OF 1,2,3,4-TETRAHYDRO-6-CARBAZOLYLCARBOXYLIC ACID



Compound	X	Y	Empirical Formula and Molecular Weight	Nitrogen, % Calcd.	Nitrogen, % Found	Melting Range, Corr., Hydrochloride	Yield, % A	Yield, % B
1	$(CH_2—CH_2)_2—N—$	$—CH_2—CH_2—$	$C_{19}H_{23}N_2O_2 \cdot HCl$ 350.68	7.99	7.94	241–242°[a]	83	62
2		$—CH_2—CH_2—CH_2—$	$C_{20}H_{25}N_2O_2 \cdot HCl$ 390.71	7.17	7.11	211–212°	82	42
3	$(CH_2—CH_2—CH_2—CH_2)_2—N—$	$—CH_2—CH_2—CH_2—$	$C_{23}H_{33}N_2O_2 \cdot HCl$ 420.76	6.66	6.64	201–202°	92	37
4	$(CH_2—CH_2—CH_2—CH_2)_2—N—$	$—CH_2—CH_2—CH_2—$	$[C_{23}H_{33}N_2O_2]_2H_2SO_4$ 806.67	6.47	6.38	109–110° Sulfate	..	..
5		$—CH_2—CH_2—CH_2—$	$C_{23}H_{31}N_2O_3 \cdot HCl$ 378.68	7.39	7.41	223–224°	73	34
6	$(CH_2—CH_2)_2—N—$	$—CH_2—CH_2—CH(CH_3)—$	$C_{23}H_{31}N_2O_2 \cdot HCl$ 378.71	7.40	7.38	187–188°	72	32

[a] Burtner reported a melting point of 234°.

TABLE II.—POTENCY, TOXICITY, AND IRRITANT EFFECTS OF 1,2,3,4-TETRAHYDRO-6-CARBAZOLYLCARBOXYLATES[a]

Compound	Solution, %	Anesthesia Duration, Min. Rabbits' Eyes	Irritation: Rabbits' Eyes	Irritation: Rabbits' Skin	LD_{50} ± S. E. Intravenous White Mice Mg./Kg.
1[b]	0.1	45	Mild	Moderate	33 ± 1.0[c]
2	0.1	43	Negligible	Slight	12.9 ± 1.1[d]
3	0.1	48	None	Mild	17.6 ± 2.39
4	0.125	28	Negligible	Moderate	31.5 ± 1.91
5	0.2	23	None	Mild	53.0 ± 2.92
6	0.1	46	Negligible	Moderate	11.3 ± 0.58
(I) Metycaine	2.0	22	Negligible	Slight	26.4 ± 4.0
(II) Butyn	2.0	19	Negligible	Slight	12.0 ± 1.0
(III) Nupercaine	0.1	30	Negligible	Slight	6.0 ± 1.0

[a] The author is indebted to Dr. K. K. Chen, Dr. C. L. Rose, and Mr. R. C. Anderson, of the Lilly Research Laboratories, for permission to reproduce these data, and wishes to acknowledge many valuable suggestions made by this group in the course of this investigation.

[b] Burtner reported twelve minutes of full corneal anesthesia in the rabbit at 0.1% concentration, twenty-nine minutes of deep corneal anesthesia at 0.5%, and an average subcutaneous MLD_{50} in mice as 80 mg./Kg.

[c] Subcutaneous toxicity in white mice, 178 ± 13.0 mg./Kg.

[d] Subcutaneous toxicity in white mice, 76.0 ± 6.9 mg./Kg.

I = γ-(2-Methyl piperidino)-propyl benzoate hydrochloride, Lilly.
II = γ-Dibutylamino-propyl-ρ-aminobenzoate-N-sulfate, Abbott.
III = Dibucaine or 2-butoxy-4-(β-diethylaminoethylamido)-carboxyquinoline hydrochloride, Ciba.

TABLE III.—BACTERICIDAL ACTIVITY OF 1,2,3,4-TETRAHYDRO-6-CARBAZOLYLCARBOXYLATES

Compound	Concentration Killing in 10 But Not 5 Min.		Phenol Coefficient, *S. aureus*		Concentration Killing in 10 But Not 5 Min.		Phenol Coefficient, *E. typhosa*	
	20°	37°	20°	37°	20°	37°	20°	37°
Phenol	1:70	1:90	1	1	1:80	1:120	1	1
2	1:200	1:600	3	7	1:1,600	1:2,000	20	17
3	1:300	1:400	4	5	...	1:1,200	..	10
6	1:150	1:400	2	5	1:800	1:2,000	10	16
Surfacaine HCl[a]	1:250	1:300	3	3	1:1,500	1:2,400	18	20

[a] Cyclomethycaine, Lilly.

esters of either of these acids was that of Horenstein and Pahlicke (4).

Method A.—The desired dialkylaminoalkyl or heterocycloalkyl chloride hydrochloride was dissolved in cold water, treated with a slight excess of sodium bicarbonate, the reaction mixture cooled, and the liberated oily liquid extracted with ether. The combined ethereal solution of aminoalkyl chloride was kept chilled at about 0° and dried with anhydrous sodium sulfate. After drying, the sulfate was removed by filtration and the solution used immediately for the preparation of the desired ester by adding and dissolving the molar equivalent of acid. The mixture was allowed to warm to room temperature and crystallization of the ester-hydrochloride usually commenced within a few minutes. In other instances, heating, removal of part of the ether, and the addition of dry hydrogen chloride was required to induce crystallization.

Method B.—Method B, used for the preparation of the ester-hydrochlorides, consisted of dissolving the acid in warm benzene, adding a molar equivalent of the dialkylaminoalkanol or heterocycloalkanol, and saturating the mixture with dry hydrogen chloride. The mixture was refluxed for about two hours with provision for the removal of water from the reaction mixture. This method produced some decomposition, and the crude ester-hydrochloride obtained required considerable purification before removal of the contaminants was effected.

Alcoholysis of the methyl ester with an excess of any of the dialkylaminoalkanols or heterocycloalkanols and attempted preparation by means of the impure acid chloride furnished products which were grossly contaminated. In all trials no success was achieved in purification and isolation of the desired ester. Although Burtner implied that he might have obtained his ester by use of the acid chloride, we have not succeeded in isolating the acid chlorides of the tetrahydrocarbazolylcarboxylic acids investigated in sufficient purity to use for preparative purposes.

All the ester-hydrochlorides prepared were readily purified by recrystallizing from hot 99% isopropanol with the aid of 0.3% w/v activated charcoal as a decolorizing and filter medium. All ester-hydrochlorides were dried over anhydrous calcium chloride for at least forty-eight hours.

3-(2-Methylpiperidino)-1-propyl 1,2,3,4-Tetrahydro-6-carbazolylcarboxylate Hydrochloride (Compound 2).—This ester-hydrochloride was readily prepared by method A (yield, 82%) and less readily by method B (yield 42%). 1-Chloropropyl-3-(2-methylpiperidinium) chloride was prepared by treating the freshly distilled alcohol ($n_D^{25} = 1.4750$) in cold dry chloroform with thionyl chloride. After completion of the reaction (about two hours), the chloroform was removed by distillation and the excess thionyl chloride dissipated by treatment with ethanol.

Compound 2 was obtained as fine white needles with a slight characteristic odor.

3-(di-*n*-Butylamino)-1-propyl 1,2,3,4-Tetrahydro-6-carbazolylcarboxylate Hydrochloride (Compound 3).—The impure ester-hydrochloride did not crystallize readily, but the purified compound was readily crystallized in fine, white needles.

3-(di-*n*-Butylamino)-1-propyl 1,2,3,4-Tetrahydro-6-carbazolylcarboxylate Sulfate (Compound 4).—The purified ester-hydrochloride (compound 3) was dissolved in warm water (1:25) and the basic ester liberated as a light yellowish oil by the addition of a slight excess of sodium hydroxide. The liberated ester was extracted with ether and the combined ester extract washed free from alkali with distilled water. The ethereal solution of the ester was chilled in the refrigerator and an equal volume of distilled water containing 0.06475 ml. of concentrated sulfuric acid for each gram of the hydrochloride employed was added and agitated. The aqueous portion was separated, the ether washed with distilled water, and the combined aqueous solution evaporated to dryness at reduced pressure. The residual salt was crystallized from hot isopropanol.

3-(4-Morpholinyl)-1-propyl 1,2,3,4-Tetrahydro-6-carbazolylcarboxylate Hydrochloride (Compound 5).—4-(γ-Hydroxypropyl)morpholine was prepared by placing 87 Gm. of freshly distilled morpholine in a three-necked flask provided with a mechanical stirrer, reflux condenser, and a dropping funnel. To the morpholine was added 105 Gm. of sodium bicarbonate and 0.3 Gm. of sodium iodide. This mixture was heated and stirred while 94.5 Gm. of trimethylene chlorohydrin was added slowly from a dropping funnel. After this addition had been completed, the mixture was refluxed for sixteen hours with constant stirring. The reaction mixture was then cooled and filtered. The residue on the filter was dissolved in 500 ml. of distilled water and the upper oily layer decanted and added to the filtrate. The latter was then fractionated and 3-morpholinopropanol was collected at 134–135° at 23 mm. Hg.[3]

Yield, 82%; $n_D^{25} = 1.4751$; $d_{25}^{25} = 1.0453$.

[3] Gardner, J. H., and Haenni, E. O., *J. Am. Chem. Soc.*, **53**, 2763(1931), reported the preparation of this alcohol by a different method and gave b. p., 134–136° at 24 mm. Hg. However, Cheney, L. C., and Bywater, W. G., *ibid.*, **64**, 970 (1942), reported a boiling point of 147–149° at 21 mm. Hg.

The 3-morpholinopropyl ester-hydrochloride was obtained as white crystals which, in contrast to the other ester-hydrochlorides, were readily soluble in cold water.

1-Methyl-3-diethylaminopropyl 1,2,3,4-Tetrahydro-6-carbazolylcarboxylate Hydrochloride (Compound 6).—Yield by method A, 72% and by method B, 32%. White needles.

SUMMARY AND CONCLUSIONS

1. Four new aminoalkanol ester-hydrochlorides and one ester-sulfate of 1,2,3,4-tetrahydro-6-carbazolylcarboxylic acid have been prepared, characterized, and studied for local anesthetic activity, toxicity, and irritation.

2. All are potent local anesthetics when applied topically, but one has to guard against the use of concentrated solutions, as these are quite irritating.

3. The topical anesthetic activity of these ester-salts is comparable to that of dibucaine hydrochloride, but they are more stable in solution and display much lower toxicities.

4. The di-*n*-butylaminopropyl ester appeared to be the most desirable for effecting anesthesia by topical application, and possibly could be used for infiltration anesthesia.

5. The 3-morpholinopropyl ester-hydrochloride was the most soluble and least toxic, but was also a less potent anesthetic.

6. Other modifications of the esterified aminoalkyl group such as increasing the chain of of the alkyl from ethyl to propyl, introducing branching in the alkyl, or varying the groups linked with the amino nitrogen, did not significantly alter the potency, toxicity, and irritant qualities.

7. These anesthetics do not interfere with the action of sulfa drugs and their inherent germicidal action is quite desirable.

8. The local anesthetic activity of di-alkylaminoalkyl esters of the isomeric acid with the carboxyl group linked at position 8 is lacking or of a very low order.

9. The aminoalkyl esters of either acid are capable of effecting neurotropic relaxation of smooth muscle.

10. Despite the high anesthetic activity of esters of the 6-acid, the irritant effects, toxicity, and limited solubility in the presence of salts might limit their utility in the local anesthetic field.

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American Association for the Advancement of Science—Pharmacy Subsection Meeting

The next meeting of the American Association for the Advancement of Science will be held in Philadelphia on December 26–31, 1951. The program of the Pharmacy Subsection of the Medical Sciences is being made up. Six sessions of this Subsection will be held. Members are invited to attend the meetings of the Section on Medical Sciences, Subsection on Medicine, and the Section on Chemistry.

Arrangements will be made for advance registration. Advance registrants will be sent a copy of the General Program before the meeting. See *Science* or *Scientific Monthly* in late August or September.

Authors who wish to present papers before the Subsection are requested to send in titles of papers *as soon as possible*. All titles for inclusion in the printed program must be in the hands of Glenn L. Jenkins, Chairman, Subsection Np, Purdue University, School of Pharmacy, Lafayette, Ind., by *September 15*.

A Comparative Study of North Dakota Fennel Oils*

By C. E. MILLER†

A report on the physical and chemical properties of the oils is presented. The use of maleic anhydride adducts as a characterizing procedure has been investigated. Fenchone is the principal ketone. The possible effect of soil composition on yield of ketone is noted.

THIS PAPER is the first in a series which will describe experimental work in volatile oil production which has been under way for several years in North Dakota. The fennel oils used in this study were obtained from the whole plant of *Foeniculum vulgare* which was grown on the experimental farm at North Dakota Agricultural College. The 1949 crop of 625 lb. was harvested from an area of 4,800 square feet, representing a yield of 5,667 lb. per acre. The plants were harvested in the flowering stage just before heavy frost (about September 25), partially cured in the field, and then steam-distilled. Approximately 200-lb. charges were used per run and the distillation was completed in about five hours. No cohobation of the aqueous distillate was carried out because of mechanical difficulties. The 1949 crop yielded 384 Gm. of oil, corresponding to a yield of 0.24 per cent on a moisture-free basis. The 1948 crop was comparable in yield, although a different plot of ground was used.

EXPERIMENTAL

Physical Properties.—The two samples of oil were light green in color. The odors were sweet and strong, resembling that of anethole, and the odor of the 1948 crop seemed more pronounced. The marked difference in congealing points and per cent of ketone of the two oils may possibly be attributed to differences in soil composition. The physical constants are recorded in Table I.

Chemical Properties.—The saponification value, ester value, iodine value, and per cent ketone were determined on the oil samples following the official procedures as outlined in the U. S. P. XIII (1) and N. F. VIII (2). The absence of free alcohols was demonstrated by negative reactions with acetyl chloride and phenyl isocyanide. Phenols, if present, could not be detected by the usual treatment with approximately normal potassium hydroxide solution or with ferric chloride. The oil decolorized a solution of bromine in carbon tetrachloride with no detectable liberation of hydrogen bromide. The results of the several chemical assays are recorded in Table II.

TABLE I.—PHYSICAL CONSTANTS

	1948 Oil	1949 Oil
Specific gravity, 25°	0.8902	0.8833
n_D^{25}	1.4984	1.4918
α_D^{25}	+40.5°	+57.6°
Congealing temperature	−7.5°	−18.5°
Solubility in 90% alcohol	4 volumes	4 volumes

TABLE II.—CHEMICAL CONSTANTS

	1948 Oil	1949 Oil
Saponification value	14.6	14.7
Ester value	14.3	14.3
Acid value	0.27 cc.	0.23 cc.
Iodine value	230.5	233.5
Per cent ketone[a]	6.0	1.5

[a] Due to a reorganization of plots at the experimental farm, the 1949 fennel crop was grown in a somewhat more alkaline soil. Whether this factor is responsible for the reduction of the ketone content of the oil is not known.

Distillation Analysis.—Samples of 50 cc. each of the oils were distilled in a Todd Precise Fractionation Assembly with spiral band column packing, using total reflux and partial take-off. Fractions were collected from 75–125°; 125–150°; 150–170; 170–200°; and a residue above 200°. Identification of the constituents in the several fractions was accomplished[1] by means of maleic anhydride adducts (3), nitrosochloride derivatives (4), and crystalline bromides (5). The results of this part of the study are given in Table III.

Ester Identification.—Very little information is available concerning the presence of esters in fennel oil (6). Since our interest in the constituents of the oil is from an economic standpoint, it seemed worth while to identify the ester or esters. Ammonolysis of esters by the use of benzylamine has been recently reported as a valuable aid in analytical procedures (7). Accordingly, isolated fractions of the oils as described under distillation analysis were treated with benzylamine in the presence of ammonium chloride as a catalyst. In each case the desired N-substituted benzylamide could not be isolated and it was concluded that a high molecular weight ester must be present.

An alternative method involving hydrolysis of the original oil with alcoholic potassium hydroxide was

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[1] Acknowledgment is given to Mr. Melvin Rubin for assisting in preparing the derivatives used in identifying the hydrocarbons and anethole.

TABLE III.—CONSTITUENTS IN 1948–1949 FENNEL OILS

Fraction, °C.	Maleic Anhydride		Nitrosyl Chloride		Bromide	Compound
	M. P., °C.	Compound	M. P., °C.	Compound	M. P., °C.	
75–125	152	2,3-Dimethyl butadiene, 1,3	...	...	...	...
125–150	128					
150–170	169	α-Pinene	85	α-Pinene	...	...
170–200	62	α-Terpinene	...	...	...	...
	83	Ocimene	103	*d*-Limonene	104	*d*-Limonene
>200	...	...	...	...	107	Anethole

tried. After working up the oil in the usual manner, an acid amide was isolated, m. p. 225°, which has been tentatively identified as benzylmalonic acid. An unreported phenylurethane, m. p. 272°, was obtained from a pyridine-catalyzed reaction mixture. The high melting point and use of pyridine (8) would indicate the presence of a phenolic hydroxyl group. A subsequent paper will discuss the identification of this phenylurethane.

Ketone Identification.—The sodium sulfite addition product obtained from the ketone determination was decomposed in alkaline medium, washed free of alkali, and dried over anhydrous sodium sulfate. The oily residue had a very pronounced odor of fenchone. Attempts to prepare the oxime and substituted hydrazones were unsuccessful.

SUMMARY

1. A comparative study of fennel oil samples from flowering plants grown during two successive years has been made. The oils are not considered to be of U. S. P. quality.

2. The properties of these oils are quite similar to those of a sample of wild fennel distilled from the stalks, flowers, and leaves and reported by Guenther (5) as having been distilled before seed formation.

3. The use of maleic anhydride adducts for the identification of terpene hydrocarbons has been successfully applied to fennel oil. It is felt that more information on terpene-maleic anhydride adducts is needed.

4. The difference in soil composition as a factor affecting the ketone content of the oils has been postulated.

5. An unidentified phenolic ester of benzylmalonic acid has been characterized.

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☆

WHO MAKES IT?

The National Registry of Rare Chemicals, Armour Research Foundation, 33rd, Federal and Dearborn Streets, Chicago, Ill., seeks information on sources of supply for the following chemicals:

Iron dithionate
Cyclohexyl isovalerate
trans-1,2-Cyclopentanedicarboxylic acid
1,4-Diamino-2-methylbutane
Pentachlorobenzaldehyde
4′-Methyl-2,4-diaminoazobenzene
2,3,4-Trichlorobenzaldehyde
2,3,4,6-Tetrachlorobenzaldehyde
Salicylalazine
Copper selenide
Pyrene-2,10-quinone
N-Phenyl-N-benzyl urethane
1,3-Di-(1-naphthyl)guanidine
cis-9,10-Dicarboxydecalin
2,8-Chrysoquinone
p-Chlorophenylmercapturic acid
Methyl stearyl ketone
Hydroxycitric acid
Trypaflavin
Cardol

A Method for the Separation of *Alpha*-Tocopherol from the Non-*Alpha*-Tocopherols*,†

By D. G. CHAPMAN, P. LICHON, and J. A. CAMPBELL‡

A direct method for evaluating the α-tocopherol content of a tocopherol concentrate is proposed. A 2 per cent petroleum ether solution of mixed tocopherols is prepared and the method is based on the removal of the non-α-tocopherols by washing with sulfuric acid in the presence of oil. The α-tocopherol remaining is titrated with ceric sulfate. The method is simple and rapid, and yields results which are in excellent agreement with those obtained by the difference method using the dipyridyl and nitrosation reactions. Factors affecting the method are discussed.

FOUR tocopherols, α-, β-, γ-, and δ-, are known to exist in nature and therefore may be present in varying amounts in different tocopherol concentrates. Of these four tocopherols only α- is credited with appreciable biological activity and hence is the most important component. The Emmerie and Engel procedure (1) based on the use of α, α′-dipyridyl is probably the most widely used procedure for the determination of total tocopherols. Another method for the determination of total tocopherols is the titrimetric technique developed in the Hoffmann-LaRoche Laboratories by Kofler using ceric sulfate (2). The non-α-tocopherols may be determined by Quaife's nitrosation reaction (3). To obtain the α-tocopherol content of an unknown, the non-α-tocopherols are subtracted from the total tocopherol content. Since the α-tocopherol content could be obtained only by the difference between two independent, time-consuming methods, the present study was initiated to ascertain if a more direct and simpler method for the estimation of α-tocopherol could be found.

The effect of sulfuric acid on tocopherols has been studied in several laboratories. Parker and McFarlane (4) reported that certain substances, which interfere in the Emmerie-Engel determination (1) of vitamin E in wheat germ oil, could be removed by washing with 85 per cent sulfuric acid. These authors found that there was no appreciable loss of added α-tocopherol. Hove and Hove (5) have studied the use of the 85 per cent (by weight) sulfuric acid reagent in the determination of vitamin E. They have shown that pure solutions of α-, β-, and γ-tocopherols are unaffected by this treatment. They have shown, further, that in the presence of 1 per cent of oil, the recovery of α-tocopherol is still 100 per cent but the recovery of β- and γ-tocopherols was unsatisfactory.

Preliminary experiments in this laboratory indicated that, when 85 per cent sulfuric acid was used to wash a petroleum ether solution of type IV-34,[1] the non-α-tocopherols were more easily removed than α-tocopherol. This observation suggested a possible method of separating α-tocopherol from the non-α-tocopherols.

METHOD

Reagents

Petroleum Ether.—Wash reagent grade benzin, Merck and Co. (boiling range 30–60°), twice with concentrated sulfuric acid and redistill.

Absolute Ethyl Alcohol.—Redistill in the presence of a small amount of potassium permanganate and sodium hydroxide pellets.

Sulfuric Acid, Specific Gravity 1.767.—Dilute reagent grade sulfuric acid until a specific gravity reading of 1.767 is obtained. Check specific gravity each day just prior to use and adjust if necessary.

Sesame Oil.—Use U. S. P. grade.

Diphenylamine Indicator.—Prepare 0.17% solution in concentrated sulfuric acid.

Ceric Sulfate, 0.01 *N*.—Prepare 0.01 *N* solution by dissolving 4.8 Gm. of $Ce(HSO_4)_4$ in 200 cc. of water, adding 28 cc. of sulfuric acid and making to 1,000 cc. with water. Standardize daily against 0.01 *N* hydroquinone.

Hydroquinone, 0.01 *N*.—Prepare daily, as required, a 0.01 *N* solution by dissolving 275.2 mg. in distilled water and making to 500 cc.

Procedure

Prepare a 2% solution of type IV concentrate in washed petroleum ether by weighing 2.00 Gm. and making to 100 cc.

Determination of Total Tocopherols.—Transfer 1 cc. of the 2% solution by pipette to a 50-cc. Erlenmeyer flask containing 15 cc. of redistilled ethyl alcohol. Add four drops of diphenylamine indi-

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[1] A concentrate of vegetable oils containing 340 mg./Gm. of total tocopherols, of which half is α- and half a mixture of β-, γ-, and δ-tocopherols. This was obtained from Distillation Products Industries, Rochester, N. Y.

cator and titrate the tocopherols with 0.01 *N* ceric sulfate to a blue end point which persists for ten seconds.

Calculation:

$$\text{Cc. of 0.01 } N \text{ ceric sulfate} \times 2.106 \times \frac{100}{2} = \text{mg. of total tocopherol per Gm. of type IV}$$

Calculation of Factor 2.106.—One cubic centimeter of 0.01 *N* ceric sulfate corresponds to 2.15 mg. of α-tocopherol, 2.08 mg. of β- or γ-tocopherols and 2.01 mg. of δ-tocopherol (2). In actual titrations with pure tocopherols the average values were found to be 2.137 for α-, 2.080 for β- and γ-, and 1.998 for δ-tocopherols. Since only a very limited quantity of the pure tocopherols was available, it was not possible to recheck these values. However, as they were in close agreement with the theoretical values, the use of the theoretical values was felt to be fully justified. According to information supplied by the manufacturer, type IV concentrate contains 340 mg. of tocopherols per gram made up of 50% α- and 50% non-α-tocopherols. The non-α-tocopherol fraction is composed of about 30% β-, 45% γ-, and 25% δ-tocopherols. Using these percentages, the factor 2.106 was obtained for use in the calculation of total tocopherols.

Determination of α-Tocopherol.—Pipette 10 cc. of the 2% type IV solution into a 30-cc. glass-stoppered centrifuge tube. Add exactly 0.15 cc. of sesame oil and mix thoroughly. Add 2 cc. of sulfuric acid solution (specific gravity 1.767) and shake the tube vigorously for three minutes. Centrifuge the tube for five minutes to separate the two layers completely. Pipette 1 cc. of the upper layer into 15 cc. of ethyl alcohol in a 50-cc. Erlenmeyer flask and titrate as before with ceric sulfate.

Calculation of α-tocopherol:

$$\text{Cc. of 0.01 } N \text{ ceric sulfate} \times 2.15 \times \frac{100}{2} = \text{mg. of α-tocopherol per Gm. of type IV}$$

The factor 2.15 is the value employed to convert cc. of 0.01 *N* ceric sulfate to mg. of α-tocopherol as pointed out above.

EXPERIMENTAL

The experiments which led to the adoption of the above procedure are discussed in the following sections. The reliability of the method in isolating the α-tocopherol fraction of the concentrate was tested by means of Quaife's nitrosation reaction, by ultraviolet absorption studies, and by the recovery of added pure tocopherols. The effect of various factors on the separation of the tocopherols is also presented.

Ceric Sulfate Titrimetric Technique.—Because of its simplicity, the ceric sulfate titrimetric technique was investigated for possible use in estimating the tocopherol content of type IV concentrate of vegetable oils. This method has been used by Kofler (2) for the estimation of pure tocopherols, but to the authors' knowledge has not been applied to vegetable oil concentrates. A concentrate of vegetable oils was analyzed by the α,α′-dipyridyl method and found to contain 344 mg./Gm. of total tocopherols. Solutions varying in concentration from 1 to 6% of this oil in petroleum ether were then prepared. One cubic centimeter of each of these solutions was added to 15 cc. of ethyl alcohol in 50-cc. Erlenmeyer flasks and titrated against 0.01 *N* ceric sulfate using diphenylamine as the indicator. The results, shown in Table I, indicate that the values for total tocopherols obtained by the ceric sulfate titration are equal to those given by the α,α′-dipyridyl procedure, provided that the concentration of tocopherol in solution is within the range of 2 to 6%. All values in this and succeeding tables are the averages of at least two determinations.

TABLE I.—EFFECT OF CONCENTRATION OF TYPE IV TOCOPHEROLS ON THE AMOUNTS OF TOCOPHEROL FOUND BY CERIC SULFATE TITRATION

Concentration of Type IV in Petrol Ether, Gm./100 Cc.	Tocopherols Found,[a] Mg./Gm.	Recovery, %
1.0	373	108
1.2	366	106
1.8	351	102
2.0	344	100
2.5	344	100
3.1	346	101
3.7	344	100
4.9	347	101
6.1	341	99

[a] Using the α,α′-dipyridyl method, 344 mg./Gm. of total tocopherols were found.

Ultraviolet Absorption Curves.—In an attempt to identify the tocopherols present in the washed 2% type IV solution, ultraviolet absorption curves were determined both before and after washing with sulfuric acid. The 2% solution of type IV tocopherols before treatment with sulfuric acid showed a maximum absorbancy at 296 mμ in petroleum ether. After washing, the maximum was at 298 mμ, which was also the maximum obtained for pure α-tocopherol in petroleum ether. However, it is recognized that small amounts of non-α-tocopherols, which may still be present, would not be detectable on such curves.

Quaife's Nitrosation Reaction.—In order to identify the nature of the acid-treated type IV solution, non-α-tocopherol determinations were carried out, before and after washing with sulfuric acid, using the method of Quaife (3) employing the Evelyn colorimeter. The results, shown in Table II, indicate that small amounts (5 mg.) of apparent non-α-tocopherols still remain. When the absorbancy of the colored solutions produced by these small amounts of non-α-tocopherols was determined using the Beckman Spectrophotometer, curves were obtained which were not typical of nitrosotocopherols. This type of curve is shown in Fig. 1, along with a curve for the typical reaction.

A further study of Quaife's nitrosation reaction, as it applies to the determination of the non-α-tocopherols remaining in the petroleum ether solution after washing with acid, was then carried out. Aliquots of the acid-washed petroleum ether solution corresponding to from 4 to 60 mg. of the original type IV concentrate were nitrosated and the ultraviolet absorption curves of the nitrosotocopherols were determined on the Beckman Spectrophotometer. Aliquots corresponding to from 4 to

TABLE II.—SEPARATION OF NON-α-TOCOPHEROLS FROM α-TOCOPHEROL IN TYPE IV CONCENTRATE USING THE SULFURIC ACID WASHING TECHNIQUE

Sample	Total Tocopherol by Ceric Sulfate Titration Mg./Gm.	Non-α-Tocopherols by Nitrosation, Mg./Gm.	α- by Difference, Mg./Gm.	Recovery of α-, %
1. Before treatment	336	168	168	...
After treatment	169	5	164	97.6
2. Before treatment	340	170	170	...
After treatment	172	5	167	98.2

30 mg. of type IV showed absorption curves which were typical of nitrosotocopherols, as reported by Quaife (3), while amounts of 40 and 60 mg. gave atypical curves and lower absorbancy readings than did the 30-mg. aliquot, as is shown in Fig. 1. Quaife (3) has stated that an aliquot containing at least 0.25 mg. of non-α-tocopherols is required for the nitrosation reaction. Therefore, in such a sample which still contained 5 mg. of non-α-tocopherols per Gm., an aliquot corresponding to 50 mg. $\left(\frac{1,000}{5} \times 0.25\right)$ of the original concentrate would be required to provide this amount of 0.25 mg. of non-α-tocopherol. However, if amounts corresponding to more than 40 mg. of type IV were used, the curves became atypical, as shown in Fig. 1. In view of this, it was felt that the nitrosation could not be satisfactorily applied to acid-washed type IV tocopherols in petroleum ether solution when a small amount of non-α-tocopherol is present in a relatively large amount of oil.

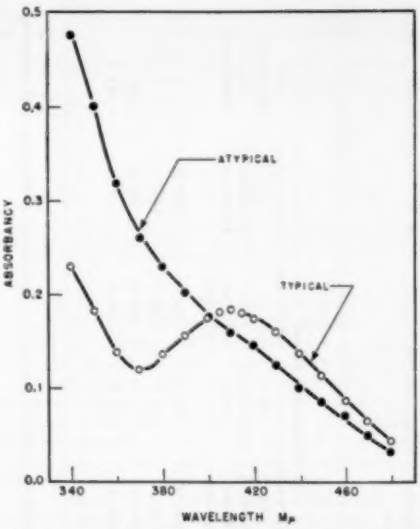

Fig. 1.—Typical and atypical nitrosotocopherol curves.

After treatment with sulfuric acid, aliquots corresponding to from 4 to 30 mg. of concentrate gave typical curves, while aliquots of 40 to 60 mg. gave atypical curves.

Effect of Sulfuric Acid Washing on Pure Tocopherols.—A study was then made of the effect of the sulfuric acid washing technique on the recovery of γ-, δ-, and α-tocopherols added to a 2% type IV solution. As is shown in Table III, four different 2% solutions of type IV were analyzed both before and after treatment with sulfuric acid. The analysis for total tocopherols by the ceric sulfate method showed values ranging from 324.3 to 330.1 mg./Gm. of concentrate (Table III, column A). Using the nitrosation reaction the non-α-tocopherol fraction was found to be one-half of the total and hence, by difference, the α-tocopherol fraction was also one-half of the total. Small amounts of pure γ-, δ-, and α-tocopherols were added to these solutions, which were again analyzed both before and after washing with sulfuric acid. When 32.25 mg. of pure γ-tocopherol per Gm. of type IV concentrate was added, bringing the total tocopherol content to 358.95 mg./Gm., on analysis, 357.1 mg./Gm. or 99.5% of the theoretical amount of total tocopherols were accounted for before washing (Table III, column H). After washing, the recovery of α-tocopherols was 100.2%. However, 0.4 mg. or 1.2% of the 32.25 mg. of added γ-tocopherol still remained in the petroleum ether solution (column K). When 14.0 mg. of δ-tocopherol was added, it was completely washed out with sulfuric acid, while there was no loss of α-tocopherol when expressed as one-half of the total. In this case 101.5% of the original plus added tocopherols were recovered before washing (column H). After washing with acid, the same tocopherol content (160.7 mg., column I) was found as was present in the original sample after washing (160.7 mg., column B).

Two lots of 30.0 and 27.5 mg. (Table III, column E) of pure α-tocopherol per Gm. of type IV concentrate were then added. The recoveries of the added plus the original tocopherols were 101.0 and 100.7% (column H). These solutions were washed with sulfuric acid and the tocopherol content again determined. The recoveries of total tocopherols, expressed as α-, amounted to 98.6 and 97.8% (column J). This indicates a loss of only 1.4 to 2.2%, which represents the precision obtainable with this method. However, when the same differences are expressed as a percentage of the added α-tocopherol, it is obvious that the recovery becomes proportionately lower, i.e., 91.0 and 84.7%.

Effect of Acid Concentration.—The effect of the concentration of acid used to wash the 2% solution of type IV tocopherols in petroleum ether was examined. Ten cubic centimeters of this solution was added to each of a series of 30-cc. centrifuge tubes. An aliquot (0.15 cc.) of sesame oil was added to each tube and the contents well mixed. Two cubic centimeters of sulfuric acid solutions which varied in specific gravity readings from 1.749 to 1.833 (25°/15°) was added. The results of this study are shown in Table IV. Using a sulfuric acid solution having a specific gravity reading of 1.767, it was

TABLE III.—THE EFFECT OF WASHING WITH SULFURIC ACID ON THE REMOVAL OF α-, γ-, AND δ-TOCOPHEROLS WHEN ADDED TO A 2% TYPE IV CONCENTRATE OF VITAMIN E USING THE CERIC SULFATE METHOD

	Tocopherols Found					Tocopherols Found						
						Before Washing			After Washing			
	(A)	(B)	(C)	(D)	(E)	(F)	(G)	(H)	(I)	(J)	(K)
Aliquot	Before Washing, Mg./Gm.	After Washing, Mg./Gm.	Theoretical α-,[a] Mg./Gm.	Recovery of Tocopherols α-, %	Total, %	Tocopherols Added, Mg./Gm.	Found, Mg./Gm.	Theoretical Total, Mg./Gm.	Recovery of Total, %	Found, Mg./Gm.	Recovery of Tocopherols,[b] %	Recovery of Added Tocopherol, %
1	320.7	163.6	163.4	100.1	50.1	32.25 γ-	357.1	358.95	99.5	164.0	100.2	1.2
2	330.1	160.7	165.1	97.3	48.7	14.0 δ-	349.2	344.1	101.5	160.7	100.0	0.0
3	324.3	162.3	162.2	100.1	50.0	30.0 α-	358.0	354.3	101.0	189.6	98.6	91.0
4	325.8	161.6	162.9	99.2	49.6	27.5 α-	355.8	353.3	100.7	184.9	97.8	84.7

[a] Since one-half of the total tocopherols is α-tocopherol, as stated by the manufacturer, and as consistently found by the difference method, this figure is obtained by dividing the total tocopherols, before washing, by two.
[b] The recoveries are expressed as α-tocopherol based on that shown in columns B plus E.

TABLE IV.—EFFECT OF THE SPECIFIC GRAVITY OF THE SULFURIC ACID SOLUTION ON THE REMOVAL OF TOCOPHEROLS[a]

Specific Gravity of Acid	Tocopherols Found, Mg./Gm.	Recovery of Tocopherols Total, %	Recovery of Tocopherols α-, %
1.749	205.7	62.3	124.6
1.764	168.5	51.0	102.1
1.767	166.2	50.3	100.7
1.772	140.8	42.6	85.3
1.808	94.5	28.6	57.2
1.833	29.9	9.1	18.1

[a] A 2% solution of type IV concentrate was used, containing 330.2 mg./Gm. of tocopherols, half of which was found to be α-tocopherol by the difference method.

found that 50% of the total tocopherols had been washed out. Sulfuric acid solutions reading less than 1.767 did not remove half of the tocopherols, while acid reading more than 1.767 gave values less than half. An aliquot of 2 cc. of sulfuric acid was chosen, as it was found that amounts up to 10 cc. did not influence further the separation of the tocopherols. This indicated that the quantity of sulfuric acid present in the reaction vessel was not critical when applied to type IV concentrate. As has been stated, type IV concentrate of vitamin E is prepared to contain 50% α-tocopherol and 50% non-α-tocopherols. Our findings in this laboratory confirm this. No other concentrates containing different proportions of α- to non-α-tocopherols were available for study.

Since the method as outlined removed practically all of the non-α-tocopherols, as indicated in Table II, and since the procedure washes out added pure non-α-tocopherols, but does not appreciably affect the α-tocopherol content as shown in Table III, it may be concluded that the remaining half of the tocopherols after washing must represent the α-tocopherol fraction. In subsequent discussion and tables, dealing with factors affecting the reaction, it becomes apparent that the condition which produces 50% recovery of total tocopherols is the condition which isolates the α-tocopherol content.

Effect of Time of Shaking.—The effect of the time of shaking on the removal of tocopherols from type IV concentrate was studied. It was found, as is shown in Table V, that shaking for a period of three minutes gave satisfactory results. Shaking for four or five minutes did not appreciably change the separation as given by the three-minute period.

Effect of Added Oil.—The effect of varying the amount of added sesame oil was also investigated. Amounts of oil from 0.10 to 0.18 cc. were added to 10-cc. aliquots of the 2% type IV solutions. This quantity of oil can be conveniently measured from a microburette or micropipette graduated to 0.01 cc. The results are shown in Table VI. From these results it would appear that the presence of a definite amount of oil is of considerable importance. This is consistent with the findings of Hove and Hove (5) who report that in a 1% oil solution α-tocopherol is unaffected by washing with sulfuric acid, while the recoveries of β- and γ-tocopherols are more or less low. These authors also found that the recoveries of added α-tocopherol (as total) averaged about 80% in the 2% oil solutions, and about 60% in the 4% oil solutions.

TABLE V.—EFFECT OF SHAKING TIME ON THE REMOVAL OF α-TOCOPHEROL[a]

Shaking Time, Min.	Tocopherols Found, Mg./Gm.	Recovery of Tocopherols	
		Total, %	α-, %
1	192.8	58.1	116.2
2	177.1	53.4	106.8
3	167.2	50.4	100.8
4	166.2	50.1	100.2
5	166.2	50.1	100.2

[a] A 2% solution of type IV was used which was found to contain 331.8 mg./Gm. of tocopherols, half of which was found to be α-tocopherol by the difference method.

TABLE VI.—THE EFFECT OF VARYING THE AMOUNT OF ADDED OIL ON THE REMOVAL OF TOCOPHEROLS FROM TYPE IV[a]

Amount of Added Oil, Cc.	Tocopherols Found, Mg./Gm.	Recovery of Tocopherols	
		Total, %	α-, %
0.10	176.4	54.4	108.8
0.12	167.7	51.7	103.4
0.15	162.3	50.0	100.1
0.18	157.7	48.6	97 2

[a] A 2% solution of type IV was used which contained 324.4 mg./Gm. of tocopherols, half of which was found to be α-tocopherol by the difference method.

Analysis of Pharmaceuticals.—Three pharmaceutical products containing type IV concentrate were examined for their total tocopherol, non-α-tocopherol, and α-tocopherol content. Total tocopherols were determined both by the α,α′-dipyridyl and by the ceric sulfate methods. The non-α-tocopherols were determined by Quaife's nitrosation reaction and the α-tocopherol both by differences and by the sulfuric acid washing technique followed by ceric sulfate titration. The results are shown in Table VII. The values obtained for total tocopherols by the two methods show close agreement.

It may be noticed in the various tables that the amount of tocopherols found both by the total assay and by the nitrosation procedure varied slightly over the period of time that this investigation was under study. Since aliquots were taken from the same opened bottle of concentrate with no precautions taken to exclude air, this discrepancy, which amounted to a maximum of 4.6% is not considered to be a reflection on the stability of the product or of any great practical significance.

The manner in which the non-α-tocopherols are removed from the petroleum ether solution with sulfuric acid in the presence of oil is not readily apparent. It appears that the non-α-tocopherols are adsorbed on the sulfuric acid-oil residue (black oily material) rather than being destroyed, since the acid-oil residue gave a tocopherol reaction. If this assumption is correct, it would suggest that the non-α-tocopherols, under the conditions of the experiment, are more readily adsorbed than α-tocopherol. This is in line with Kofler's findings (2) of the adsorption affinities of the tocopherols when using aluminum oxide as an adsorbent.

TABLE VII.—THE DETERMINATION OF THE TOCOPHEROL CONTENT OF THREE PHARMACEUTICAL PRODUCTS USING THE SULFURIC ACID WASHING TECHNIQUE

Sample	Total Tocopherols by Ceric Sulfate Method, Mg./Cap.	Total Tocopherols by Dipyridyl Method, Mg./Cap.	Non-α-Tocopherols by Nitrosation Reaction, Mg./Cap.	α-Tocopherol by Difference Based on Total by Dipyridyl Method, Mg./Cap.	α-Tocopherol by H_2SO_4 Method, Mg./Cap.	Recovery of α-% of That Found by Difference Method
A	141.0	141.0	69.6	71.4	70.0	98.0
B	51.3	50.0	24.4	25.6	25.6	100.0
C	46.1	45.8	22.8	23.0	22.4	97.4

Assuming that half of the total tocopherols is present as α-tocopherol, then the recoveries of α-tocopherol by the sulfuric acid treatment ranged from 97.4 to 100.0%.

DISCUSSION

The ceric sulfate titrimetric method has been found to be satisfactory for the determination of total tocopherols in a concentrate. The method, as outlined above, gave values very similar to those found by the α,α′-dipyridyl procedure. It has the advantage of being more rapid and simpler. It has also been found that the dipyridyl method may be used on the washed tocopherols.

The precision of the method was tested by carrying out the ceric sulfate titration after washing with sulfuric acid on ten separate weighings of approximately 2.0 Gm. of the type IV concentrate. The coefficient of variation was found to be 1.59%, indicating that the precision of the method was satisfactory. It has been found advisable, in carrying out the analysis of unknown capsules, to extract the oil from the capsules and base the preparation of the 2% solution on the weight of oil obtained. It may be expected, therefore, that the precision found above would be applicable to any unknown sample containing type IV tocopherols.

The factors such as the time of reaction and the presence of oil are quite critical. In the absence of oil, the acid-washing has no effect on any of the tocopherols. The concentration of the sulfuric acid is also critical and the specific gravity reading must be made just prior to using the solution. Even in a glass-stoppered bottle the specific gravity appears to change from day to day. When shaking the reaction tubes, vigorous agitation is required to obtain the desired results.

The method has been applied to tocopheryl acetate after hydrolysis. It has been found that under the conditions as outlined above, some of the α-tocopherol is removed by the treatment along with the non-α-tocopherols. It appears that the original oil is altered during hydrolysis to such an extent that the conditions of the method do not result in a quantita-

tive separation and therefore cannot be applied, without modification, to the acetates. Nevertheless, in view of the lack of direct methods, it was felt that this procedure furnished a simple and rapid means of determining α-tocopherol in mixtures of natural tocopherols.

SUMMARY

A method for estimating the α-tocopherol content of a vitamin E concentrate and pharmaceuticals prepared from the concentrate is proposed. The method is based on the removal of the non-α-tocopherols by washing a 2 per cent petroleum ether solution of the mixed tocopherols, in the presence of oil, with a sulfuric acid solution having a specific gravity of 1.767. After washing, the remaining tocopherol, which has been shown to be chiefly α-, is titrated against ceric sulfate.

Factors affecting the method, such as specific gravity of acid, time of shaking, and amount of oil, are critical. The method was simple and rapid and gave results which were in close agreement with those obtained by the difference method using the dipyridyl and nitrosation reactions.

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Isolation of Quercitrin and Quercetin from Lemon Flavine*

By ALBERT N. BOOTH and FLOYD DeEDS†

A simple method for the isolation of pure quercitrin and quercetin from lemon flavine in high yield is described. Application of the method to several samples of lemon flavine has given uniformly satisfactory results.

One of the questions arising in the course of investigations on the acute and chronic toxicity and mechanism of action of rutin is concerned with the role, if any, played by the glucose and rhamnose attached to the No. 3 carbon of quercetin. To answer this question, at least in part, required a comparison of the physiological effects produced by rutin, quercitrin, and quercetin, and a comparison of their chronic toxicities. For these purposes a liberal supply of pure quercitrin and quercetin was required. Evidence has been presented from this laboratory (1–4) to the effect that quercitrin and quercetin exert the same physiological effects as rutin.

The isolation of quercitrin and quercetin from goldenrod has been described by Guthrie, O'Connor, Stansbury, and Savich (5). The quercitrin used in the early investigations by this laboratory was prepared in this manner from crude material isolated from goldenrod and supplied by the Southern Regional Research Laboratory. In the search for materials from which the flavonoids could be isolated in large yield with a minimum of effort, lemon and orange flavine were investigated. In our experience, the methods of isolation described in Abderhalden's "Biochemisches Handlexikon" (6) were relatively troublesome and did not give high yields.

Lemon and orange flavines are obtained from the bark of black oak, and are produced commercially as dyestuffs. The principal flavonoid in lemon flavine is quercitrin, whereas quercetin is the principal constituent of orange flavine. Quercitrin is the rhamnoside of the aglycone quercetin. In rutin the rhamnose is attached to glucose, which replaces the hydrogen of the hydroxyl group in the 3-position of quercetin. We have found that it is much easier to obtain pure quercitrin from lemon flavine than it is to isolate quercetin from orange flavine in a comparable state of purity. For the preparation of quercetin of high purity we have, therefore, relied upon acid hydrolysis of quercitrin from lemon flavine.

The quercitrin of lemon flavine is readily soluble in 95 per cent ethyl alcohol, but other alcohol-soluble material is difficult to remove subse-

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† The microanalyses were made by M. T. Kilpatrick and the spectrophotometric data were obtained by P. Stitt of the Western Regional Research Laboratory.

TABLE I.—IDENTIFICATION DATA FOR QUERCITRIN AND QUERCETIN

Compound	Carbon, % Found	Carbon, % Calcd.	Hydrogen, % Found	Hydrogen, % Calcd.	Absorption Maxima, mμ* Found	Absorption Maxima, mμ* Reported	Specific Extinction Coefficient, L./Gm.-Cm.* Found	Specific Extinction Coefficient, L./Gm.-Cm.* Reported
Quercitrin	56.3	56.25	4.56	4.46	257.0	261.2 (8)	47.5	36.3 (8)
						260.0 (5)	...	...
						257.0 (9)	...	...
					361.0	356.0 (8)	37.5	28.9 (8)
						350.0 (5)	...	...
						353.0 (9)	...	...
Quercetin	59.6	59.6	3.48	3.31	256.0	255.5 (8)	71.0	...
						256.0 (5)	...	67.0 (5)
						256.0 (9)	...	...
						257.0 (9)	...	...
						257.5 (7)	...	75.7 (7)
					373.0	375.5 (8)	76.5	...
						372.0 (5)	...	75.5 (5)
						372.0 (9)	...	...
						373.0 (9)	...	...
						375.0 (7)	...	80.0 (7)

* 95% ethanol was used as solvent.

quently. In the hope that some mixture of alcohol and water might effect a better separation of quercitrin and other constituents of lemon flavine, the following experiment was set up. Five grams of lemon flavine was placed in each of a series of flasks to which were added 100-ml. quantities of varying percentages of alcohol, ranging from 5 to 95 per cent. The flasks were stoppered and placed on a shaking machine for thirty minutes, and then allowed to stand overnight at room temperature. Somewhat to our surprise, we noted that the product which settled out at alcohol concentrations in the range of 5 to 35 per cent looked like reasonably good quercitrin, while the other constituents remained in solution or suspension. This observation led to the development of the following procedure in which other alcohols such as methyl or isopropyl may be substituted for ethyl alcohol. A public service patent No. 2,534,250 covers this procedure.

PROCEDURE

One hundred grams of lemon flavine was placed in a 4-L. bottle and shaken thoroughly with 200 ml. of 95% ethyl alcohol, which acts as a good wetting agent and dissolves some of the flavine constituents. During constant agitation 3 L. of water was added. After thorough shaking the mixture was allowed to stand for twenty-four hours. The supernatant material was decanted, and the insoluble residue, which contained considerable mother liquor, was washed with water and sucked as dry as possible on a Büchner filter. The residue was suspended in 250 ml. of acetone and heated until all acetone-soluble material was in solution. The acetone solution was filtered while hot, and the filtrate collected in 1.5 L. of hot distilled water. The mixture was heated on a steam bath and agitated constantly until all acetone was evaporated. The aqueous suspension of quercitrin was filtered while hot and the precipitate washed with water. The product was dried at 100°. The yield was 37 Gm. A high degree of purity may be obtained by recrystallization as described above.

Quercetin was prepared from quercitrin by hydrolysis with 2% sulfuric acid heated on the steam bath for two hours. The hydrolysate was filtered while hot and the precipitated quercetin washed with distilled water, sucked dry in a Büchner filter, and finally dried at 100°. The melting point was 309°.

The purity of the isolated quercitrin and quercetin was established by determination of the carbon and hydrogen values, and by ultraviolet absorption data. For carbon and hydrogen analyses the quercitrin and quercetin were dried *in vacuo* at temperatures of 150 and 170°, respectively. The carbon and hydrogen values are the averages of several determinations. The identification data are given in Table I.

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Hog Bile Extract: An Identification Test*

By HOWARD J. ANDERSON, MARGARET TARGY, JAMES T. RUDESILL, EDSON R. DOHNER, and FRED W. WHEELER

Hog bile salts when mixed with sulfuric acid and benzaldehyde give a purple color, but this test is unsuitable for the detection of hog bile in mixtures with ox or sheep bile salts, which produce a brown color obscuring the purple color. A quantitative separation of hog bile from sheep or ox bile is described. The principle involved depends upon the action of hydrochloric acid which precipitates hog bile from solution in the form of a gummy mass, whereas with ox or sheep bile only slight precipitates are formed under the conditions of the test described.

At the present time there is apparently no published analytical method which adequately indicates the purity of a hog bile salt, or the amount of hog bile in a mixture with either ox bile or sheep bile salts. An identification test for hog bile extract is presented herewith. It consists of a qualitative color test as an exploratory procedure, and in addition, the precipitation of conjugated hog bile salts from solution of mixtures of hog and ox bile, as quantitatively as possible.

It is possible that hog bile extract might be used as a replacement, at least in part, for ox bile extract, thus liberating a large quantity of ox bile for the manufacture of cholic and desoxycholic acids for the synthesis of drugs such as cortisone.

Bile salts, along with bile pigments, mucin, cholesterol, lecithin, fatty acids, fats, and inorganic salts, are described as the chief components of bile (1–3). Ox bile and hog bile differ considerably in the kind of bile salts they contain. The principal bile acid in ox bile is cholic acid (3,7,12-trihydroxycholanic acid) (4); also present in somewhat lesser amounts is desoxycholic acid (3,12-dihydroxycholanic acid). Lithocholic and chenodesoxycholic are present in minor amounts. Both ox bile and hog bile contain their respective bile acids as the sodium salts, conjugated with taurine and glycine. Thus, ox bile contains sodium glycocholate and sodium taurocholate as the chief bile salts (5–8).

The essential bile acid of the hog is hyodesoxycholic acid (3,6-dihydroxycholanic acid); also present is 3-hydroxy-6-ketocholanic acid. The separation of hyodesoxycholic acid, the keto acid, and others, has been excellently described (9). Many other research workers have published papers on hog bile acids (10–13).

Experiments have been reported studying the effect of administering bile salts to dogs (14, 15). Thus, it has been shown that "three grams daily of desiccated hog bile caused a 38% (average) increase in the output of bile in biliary fistula dogs. This is the same as the increase obtained with three grams of ox bile salts containing from 1.5 to 1.7 grams of cholic acid." (14).

It has been said that "when hog bile preparations are used in man, as judged by results with dogs, they will produce an increase in bile volume output equivalent to that obtained with equivalent amounts of unoxidized conjugated ox-bile salts, or 'natural' ox-bile salts" (14).

We are presenting a method herein for the identification of pure hog bile salts.

EXPERIMENTAL

Color Test for Hog Bile Salts

As previously stated, a blue color test is official for the colorimetric assay of ox bile salts (U. S. P. XIV) for cholic acid content (4). For hog bile no such quantitative test is available.

However, pure hog bile salts, when mixed with sulfuric acid and benzaldehyde, under conditions of the test, give a purple color (16, 17). It would be possible, therefore, to distinguish pure hog bile from pure ox bile salts, as the latter gives a brown color. This test, however, cannot be used for mixtures of the two, as the purple color is obscured by the brown color imparted by the cholic acid present in the ox bile.

According to our plan, the purple color test could be used to determine the purity of hog bile salts, while the precipitation procedure, described later, could be used to determine the approximate amount of hog bile in mixtures.

As a qualitative indication, therefore, the purple color test will tell whether or not pure hog bile salts are contaminated with ox or sheep bile, the latter two giving brown colors.

The color test can be conducted as follows: use 20 mg. of hog bile extract, 2 cc. of 75% sulfuric acid (by volume), and 0.1 cc. of benzaldehyde. Heat the mixtures to 50° for five minutes; then add 5 ml. of glacial acetic acid; let stand at room temperature one-half hour. Then dilute 0.5 cc. of the mixture with 5 cc. of glacial acetic acid.

A purple color indicates a pure hog bile salt. If even a very small amount of ox bile is present, the characteristic purple color is obscured by a brown

* Received March 15, 1951, from the Pharmaceutical Research Laboratories, The Cudahy Packing Co., Omaha, Nebr.

color. It is interesting to note that pure hyodesoxycholic acid, prepared in our laboratories, yields a blue color; pure cholic acid gives a brown color.

Precipitation Test

Upon the addition of hydrochloric acid, hog bile will precipitate from solution, forming a large heavy gummy mass. Since beef and sheep bile form only a very small precipitate under conditions of the test, we have been using this as a means of qualitatively determining the presence of hog bile.

It was therefore expected that various mixtures of hog and beef bile would form precipitates depending in quantity on the amount of hog bile present in any mixture with beef or sheep bile, and that this procedure could be extended to become a quantitative method.

According to our plan, then, the purple color test could be used to determine the purity of hog bile extract, while the precipitation method could be used to determine the approximate amount of hog bile in mixtures.

Procedure.—Various proportions of hog and beef bile salts were mixed to total 1.0 Gm., placed in 50-cc. tared beakers, and dissolved in 20 cc. of water. To each beaker, 1 cc. of 1:1 mix (concentrated hydrochloric acid and water) was added slowly. After standing for fifteen minutes the precipitates were filtered on tared Gooch crucibles, and washed once with 2 cc.[1] of water. Both the beakers, containing some adhering precipitate, and the crucibles were dried and weighed, and the total precipitates found.

Experiments were conducted using the above procedure on U. S. P. ox bile extract salts, desiccated ox bile, hog bile extract, and desiccated hog bile.

TABLE I[a]

Hog Bile[b] (Actual), %	Wt. of Ppt. Gm.	Mean Wt. of Ppt., Gm.	Hog Bile (From Curve), %	Deviation, %
0.00	0.0218	0.0140	3.00	3.00
	0.0170		1.00	1.00
	0.0152		0.75	0.75
	0.0117		−0.50	0.50
	0.0098		−0.75	0.75
	0.0099		−0.75	0.75
	0.0124		−0.25	0.25
20.00	0.0728	0.0758	19.50	0.50
	0.0810		20.50	0.50
	0.0878		20.75	0.75
	0.0638		16.00	4.00
	0.0737		19.75	0.25
33.00	0.2100	0.2686	28.75	4.25
	0.2877		34.00	1.00
	0.2972		34.75	1.75
	0.2960		34.75	1.75
	0.2521		32.00	1.00
50.00	0.5212	0.4984	52.50	2.50
	0.4577		46.75	3.25
	0.4838		49.00	1.00
	0.5554		55.50	5.50
	0.5095		51.00	1.00
	0.5080		50.75	0.75
	0.4572		47.00	3.00
	0.4896		49.50	0.50
	0.5158		51.75	1.75
	0.5441		54.50	4.50
	0.4406		46.00	4.00
60.00	0.6080	0.6001	60.75	0.75
	0.6002		60.00	0.00
	0.5539		55.50	4.50
	0.5672		56.50	3.50
	0.6168		61.50	1.50
	0.6277		63.00	3.00
	0.6267		62.75	2.75
75.00	0.6730	0.7032	68.75	6.25
	0.7260		75.75	0.75
	0.7570		80.50	5.50
	0.6676		67.75	7.25
	0.6924		71.00	4.00
90.00	0.7830	0.7935	86.00	4.00
	0.8083		90.75	0.75
	0.7547		80.50	9.50
	0.8447		...	...
	0.7770		84.50	5.50
100.00	0.8322	0.8381	99.00	1.00
	0.8066		90.50	9.50
	0.8113		92.50	7.50
	0.8445		102.00	2.00
	0.8462		102.00	2.00
	0.8453		102.00	2.00
	0.8655		...	...
	0.8577		105.00	5.00
	0.8210		95.25	4.75
	0.8213		95.25	4.75
	0.8672		...	...

[a] Mean deviation = 2.80 ± 0.22%.
[b] Remainder ox bile.

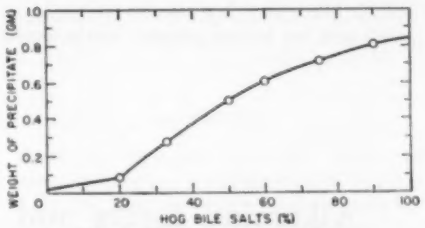

Fig. 1.—Hog bile standard curve.

Results in Table I indicate weights of precipitate, obtained by the method described, when various mixtures of different types of hog bile preparations are mixed with the various kinds of ox bile salts. Using the mean weight of the precipitates, a hog bile standard curve was devised, and we have found by running sample mixtures that we can quite closely determine the amount of hog bile salts in a mixture with other bile salts.

It will be noted from the Table I, where no hog bile is present and the sample consists entirely of ox bile salts, that the mean weight of the precipitates from seven experiments was 0.0140 Gm. Likewise, where the sample contains all hog bile and is not contaminated with any other kind of bile, the mean weight of the precipitate is 0.8381 Gm.

Similarly, a mixture of one-half hog bile salt and one-half ox bile salt yields a precipitate weighing 0.4984 Gm. (average of 11 tests). Data not presented in this paper indicate that sheep bile gives precipitates of the same ratio of magnitude as ox bile.

By the method described, any concentration of solution less than 1% hog bile content cannot be

[1] The precipitate is sticky, forms in clumps, and is difficult to wash. One 2-cc. wash produces essentially the same results as four separate 2-cc. washes.

determined, and according to the test, this means any dry mixture containing less than 20% hog bile salts. The precipitate, because of its nature, cannot be washed well. This latter defect, however, is relative, and can be taken care of by the use of the solubility curve shown as Fig. 1.

It is true the curve may vary with different lots of hog and beef bile salts, but even then the deviation is not unreasonable, as shown in the final column of Table I, when the standard curve is used.

CONCLUSION

1. An identification test is proposed for hog bile. This test, together with increasing availability and pharmaceutical use of hog bile, suggests the advisability of including hog bile extract in either the U. S. Pharmacopeia or the National Formulary. The monograph on the official product might well include: Description; Solubility; Acidity; Insoluble Substances; Qualitative Color Test—Purple Color; Assay; Quantitative Precipitation from Mixture if Not Pure; Packaging and Storage; Dosage (same as ox bile extract).

2. The proposed assay method is not for a specific bile acid in hog bile, as for hyodesoxycholic acid, but the test provides for the identity of a pure hog bile salt by a qualitative color test. Where the color test indicates the possible presence of bile salts other than those of hog bile, the precipitation method described above indicates the amount of hog bile salt, and the amount of beef and/or sheep bile present.

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Alkamine Esters and Amides of Cumic Acid*

By M. B. MOORE

A series of previously unreported esters and amides of cumic acid is presented, with appropriate physical data. None was found to surpass the β-diethylaminoethyl ester in value as a local anesthetic.

THE PUBLICATION of a paper by Bryan and Foote (1) on esters of cumic acid has prompted this report of a number of similar esters synthesized in these laboratories for testing as local anesthetics. Since the esters of this series show no advantage as local anesthetics over the diethylaminoethyl ester, their publication may prevent needless repetition of work. Included are two amides which were prepared and tested at the same time. Pertinent data are summarized in Table I.

Except for those reported by Bryan and Foote (1), the only alkamine esters of cumic acid found in the literature were included in the series by Reasenberg and Goldberg (2) in which the alkamine residue contained a secondary amine, and by McElvain and Carney (3) in which the amine was the 2-methyl-1-piperidine radical. In neither case did the cumic acid esters appear to be as effective therapeutically as those derived from some other acids.

The syntheses were carried out by a conventional method, in which the acid chloride and amino alcohol or diamine were allowed to react in dry benzene. The salts of the amides proved to be very difficult to purify, and when the results of the pharmacologic testing of these two became known, no further members were synthesized.

The pharmacologic tests were carried out by Dr. R. K. Richards and his staff. Compounds

* Received February 9, 1951, from the Organic Research Department, Abbott Laboratories, North Chicago.

TABLE I.—ESTERS AND AMIDES OF CUMIC ACID

No.	R	Yield, %	B. P.	Mm.	Hydrochloride[c] M. P.	Hydrochloride[c] Formula	Analysis, % N Calcd.	Found
1	$OCH_2CH_2N(C_2H_5)_2$[a]	40	...	...	118–121	$C_{18}H_{30}NO_2 \cdot HCl$	4.67	4.71
2	$OCH_2CH_2N(n\text{-}C_4H_9)_2$	50	...	...	105–106	$C_{22}H_{38}NO_2 \cdot HCl$	3.94	3.78
3	$O(CH_2)_2N(n\text{-}C_4H_9)_2$	62	...	...	130–131	$C_{22}H_{38}NO_2 \cdot HCl$	3.79	3.62
4	$OCH_2C(CH_3)_2CH_2N(CH_3)_2$	57.5	...	...	140–142	$C_{17}H_{27}NO_2 \cdot HCl$	4.46	4.35
5	$OCH_2C(CH_3)_2CH_2N(CH_3)_2O(CH_2)_2$	70	...	...	142–144	$C_{18}H_{29}NO_3 \cdot HCl$	3.94	3.90
6	$OCH_2CH_2NH-C(CH_3)(CH_2-O-CH_3)(CH_2-O-CH_3)$	32.5	205–207	4	...	$C_{17}H_{25}NO_4$	4.57	4.55
7	$NHCH_2CH_2N(C_2H_5)_2$[b]	63	...	...	136–137	$C_{18}H_{32}N_2O \cdot HCl$	9.38	8.84
8	$NHCH(CH_3)(CH_2)_2N(C_2H_5)_2$	30	200–218	5	158–159	$C_{18}H_{32}N_2O \cdot HCl \cdot H_2O$ $C_{18}H_{32}N_2O$	8.78 9.20	9.11

[a] Reported by Bryan and Foote (1) to have m. p. 131–132° (corr.).
[b] Sample recrystallized successively from methyl propyl ketone, benzene, di-isopropyl ketone, and soaked in alcohol.
[c] Melting points are uncorrected.

1 and 7 were approximately equal to procaine in anesthetic effect in wheals, but were irritating. The other compounds listed were in no case less irritating and usually were much more so. All except compounds 6 and 8 were reported to have local anesthetic effect.

EXPERIMENTAL[1]

γ-Di-*n*-butylaminopropyl Cumate.—γ-Di-*n*-butylaminopropanol, 9.5 Gm. (0.05 mole), in 30 cc. of dry benzene was added gradually through a dropping funnel to a stirred and refluxing solution of 9.2 Gm. (0.05 mole) cumoyl chloride in 50 cc. of dry benzene. After an hour of refluxing, no precipitation occurred. The solution was filtered and the filter washed with a little benzene; crystallization began and the filtrate became a crystal mush. The crystals were removed by filtration and dried at 100°; yield, 13 Gm. (70%). The product was recrystallized from ethyl acetate and again from benzene, m. p. 130–131°.

N-δ-Diethylamino-α-methylbutyl Cumoylamide.—Cumoyl chloride, 9.2 Gm. (0.05 mole), was dissolved in 50 cc. of dry benzene. To the stirred solution 10 Gm. (about 0.05 mole) of Novoldiamine (δ-diethylamino-α-methylbutylamine) was added dropwise to prevent too rapid heating; the product was then refluxed for three hours to assure completion of the reaction. The solvent was removed on the steam bath, the residue was taken up in water with a little excess hydrochloric acid, the aqueous solution was shaken out with ether to remove nonbasic impurities and was filtered with the aid of a little charcoal. The clear aqueous solution was made strongly alkaline; the base was shaken out in ether finally distilled after removal of the ether, b. p. 200–218° (5 mm.); yield, 5 Gm. (30%).

[1] The cumoyl chloride and compound 1 were first prepared in these laboratories by Dr. E. E. Moore. All analyses were performed by Mr. E. F. Shelberg, Chief Microanalyst, and his staff.

SUMMARY

Some new alkamine esters and amides of cumic acid have been synthesized, and their hydrochlorides have been studied as local anesthetics. The variety of different groups used has shown no member with outstanding activity devoid of irritating effects.

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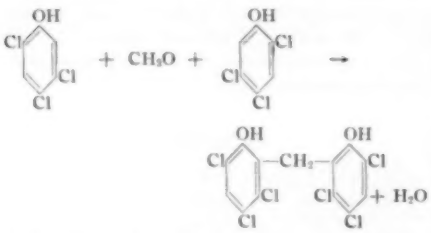
Solubility and Bacterial Studies of Hexachlorophene*,†

By ROBERT M. GREGG‡ and LOUIS C. ZOPF§

The diphenolic compound, hexachlorophene, has been shown to be a very effective germicide. Continued use of the compound in soap appears to reduce the bacterial population of the skin to as low as 5 per cent of normal. This high degree of effectiveness, coupled with a low degree of toxicity and a low sensitization index, suggest the use of hexachlorophene in dermatological pharmaceuticals. Hexachlorophene was rendered soluble by the use of Tween 20 and by Tween 80, which greatly enhances its potency. Three official ointment bases containing hexachlorophene showed no significant differences in the widths of the zones of inhibition. Corn oil, olive oil, and cottonseed oil proved to be suitable solvents for hexachlorophene. It may be solubilized in liquid petrolatum with the aid of Span 85. It is insoluble in glycerin, but is freely soluble in the closely related solvents propylene glycol and the polyethylene glycols. Hexachlorophene may prove to be adaptable for use in ointments, lotions, and antiseptic oils.

In 1939 Gump (1) applied for a patent covering the process of manufacture of the diphenolic compound, bis-(2-hydroxy-3,5,6-trichlorophenyl)-methane. This compound is frequently referred to as hexachlorophene, a generic term accepted by the Council on Pharmacy and Chemistry of the American Medical Association (2). Gump suggested its use as a bactericidal, fungicidal, and preserving agent in such substances as toothpowders, toothpastes, ointments, creams, rubber goods, etc. He prepared the compound by reacting two molecules of 2,4,5-trichlorophenol with one molecule of formaldehyde in the presence of concentrated sulfuric acid.



Hexachlorophene is a white crystalline substance having a melting point of 164–165°. It is soluble in most of the organic solvents. According to Gump, 100 cc. of ethyl alcohol, acetone, benzene, or toluene will dissolve 50 Gm., 191 Gm., 5.3 Gm., and 5.6 Gm. of hexachlorophene, respectively. Hexachlorophene is practically insoluble in water, Gump reporting the water solubility as being less than 0.001 per cent. The compound is weakly acidic in nature, forming soluble sodium and potassium salts.

Hexachlorophene is strongly bactericidal in action. Using *Staphylococcus aureus* as the test organism, the phenol coefficient of the monosodium or potassium salt is said to be about 125 when tested according to the U. S. Food and Drug Administration (3) method. It is less effective against Gram-negative bacteria, displaying a phenol coefficient of 20 against *Escherichia coli* and *Salmonella typhosa*.

To date, the greatest area of usefulness for hexachlorophene has been in soaps. Especially interesting have been the reports indicating a reduction in the amount of time needed for the preoperative scrub when hexachlorophene is incorporated with the soap. Most investigators agree that best results are obtained when hexachlorophene-containing soaps are used to the exclusion of ordinary soaps. Price and Bonnett (4) performed serial basin tests which "seemed to show" a reduction in the bacterial population of the skin to about 5 per cent of the usual number within four to seven days when hexachlorophene soap was used exclusively. Fuller, *et al.* (5), showed a significant decrease in carbuncles, large furuncles, and cellulitis when hexachlorophene soap was used exclusively for one year in a state school for the mentally deficient.

Traub, Newhall, and Fuller (6) performed patch tests on 600 individuals and found no sensitivity. Fuller, *et al.*, found that no irritation or sensitivity developed among 389 subjects using hexachlorophene soap for one year.

Gump (7) reports hexachlorophene to be relatively nontoxic to guinea pigs when given orally. Price and Bonnett, however, found it to be very toxic to dogs when given intravenously. Doses

* Received April 2, 1951, from the College of Pharmacy, State University of Iowa, Iowa City.
Abstracted from a thesis submitted to the Graduate College of the State University of Iowa by Robert M. Gregg as partial fulfillment of the requirements for the degree of Master of Science.
† The hexachlorophene used in this study was supplied by the Sindar Corporation, New York, N. Y. The Tween 20, Tween 80, and Span 85 were supplied by the Atlas Powder Company, Wilmington, Del.
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as small as 5 mg./Kg. of body weight caused convulsions, intravascular clotting of blood, respiratory paralysis, and death. They advise against its use on open wounds.

Evidence of its low sensitization index and indications of its effectiveness against skin bacteria suggested that hexachlorophene might be of value in dermatological pharmaceuticals. The one obvious limiting factor is its low water solubility. The objective of this investigation was to find an efficient, nonirritating solubilizing agent for hexachlorophene and to determine its effect, if any, on the antibacterial properties of hexachlorophene. In addition, the solubility of hexachlorophene in certain substances commonly employed in ointment bases was determined with the view of evaluating its effectiveness in vehicles of this type.

EXPERIMENTAL

Tween 20[1] and Tween 80[1] were tried as solubilizers for hexachlorophene. It was found that 0.5 Gm. of hexachlorophene is soluble in 100 cc. of a 10% solution of either of the above agents. The hexachlorophene did not dissolve immediately in the Tween solution, but complete solubility was effected upon several days' standing with occasional agitation. The solution was more readily prepared by dissolving the hexachlorophene in the Tween with the aid of the water bath and then adding water to bring the solution to the desired volume. If a minimum of 12 parts Tween is used to one part hexachlorophene, any amount of water may be added without precipitating the hexachlorophene. Dilutions were made, and hexachlorophene solutions of 1:200, 1:1,000, and 1:2,000 were stored at 35–37°, 8–10°, and at room temperature to test for stability.

Using a twenty-four-hour broth culture of *Staphylococcus aureus*, the Ostrolenk (8) total swirl method of germicidal testing was modified to determine the antibacterial potency of the Tween 80-hexachlorophene solution. One modification consisted of the use of standard F. D. A. broth throughout. Ostrolenk carried the organism in 2% trypticase broth and incubated the culture-medication mixture in fluid thioglycollate broth. In this study both five- and ten-minute culture-medication contact periods were employed, whereas Ostrolenk used only the former. It was thought that the two would yield a more accurate end point than would the single five-minute period.

Aqueous hexachlorophene-Tween 80 solutions were prepared in which the ratio of the amounts of Tween to hexachlorophene was 14:1. Dilutions for germicidal testing were made from these stock solutions and sterilized with steam under pressure to eliminate any hexachlorophene-resistant species which may have been present. Phenol solutions in concentrations of 1:70, 1:75, and 1:80 were employed as standards.

The phenol coefficient was determined by dividing the denominator of the fraction, expressing the dilution of hexachlorophene killing *Staphylococcus aureus* in ten minutes contact but not in five minutes, by the denominator of the phenol dilution fraction exhibiting the same killing power.

The solubility of hexachlorophene in several commonly employed ingredients of ointment bases was determined. It was found to be freely soluble in propylene glycol, polyethylene glycol 400, and in white wax. It is relatively insoluble in glycerin and white petrolatum. One part of hexachlorophene failed to dissolve in 1,000 parts of either of these agents. Hexachlorophene was found to be freely soluble in corn oil, olive oil, and cottonseed oil. Although the compound is relatively insoluble in liquid petrolatum, it can be placed in solution in this liquid by dissolving it in four parts of Span 80[2] and adding this solution to the liquid petrolatum.

Hexachlorophene was incorporated with the following official ointment bases:

Hydrophilic Ointment, U. S. P. XIV.—This ointment normally contains 37% water, which for this study was replaced by an aqueous solution of 2.7% hexachlorophene and 32.4% Tween 80. This results in an oil-in-water emulsion of slightly higher consistency than the official base. The finished ointment contains 1% hexachlorophene and 12% Tween 80.

Hydrophilic Petrolatum, U. S. P. XIV.—The hexachlorophene was dissolved in the melted white wax and the remainder of ingredients incorporated in the usual manner.

White Ointment, U. S. P. XIV.—The hexachlorophene was dissolved in the melted white wax. The white petrolatum was incorporated in the usual manner.

Polyethylene Glycol Ointment, U. S. P. XIV.—The polyethylene glycol 400, polyethylene glycol 4000, and hexachlorophene were heated together on a water bath until fusion and solution were complete. The solution was allowed to congeal with stirring.

The four ointments were prepared with 1% hexachlorophene. Their bactericidal potencies were determined by a modified F. D. A. agar cup-plate method. *Staphylococcus aureus* was again employed as the test organism. The modification concerned the method of introduction of the ointment into the cups. For this purpose a standard 10 cc. Luer hypodermic syringe was modified by removing the glass tip and shoulder, leaving a cylinder of 15-mm. inside diameter. The ointment was placed in the cups by means of this instrument and disengaged from the plunger with the aid of a warmed spatula. The following bases were used as controls:

(*a*) Hydrophilic Ointment, U. S. P. XIV
(*b*) Hydrophilic Ointment, U. S. P. XIV (32.4% Tween 80 solution used in place of water)
(*c*) Hydrophilic Petrolatum, U. S. P. XIV
(*d*) White Ointment, U. S. P. XIV
(*e*) Polyethylene Glycol Ointment, U. S. P. XIV

The plates were incubated forty-eight hours and the zones of inhibition measured. The results of these tests are shown in Table I and Figs. 1–4.

[1] Trade marks of Atlas Powder Co. for polyoxyethylene sorbitan monolaurate and polyoxyethylene sorbitan monooleate respectively.

[2] Trade mark of Atlas Powder Co. for sorbitan trioleate.

DISCUSSION

If hexachlorophene is to be used in aqueous dermatological pharmaceuticals, a bland agent must be used as a solubilizer. The low irritating properties of the nonionic Tweens, as well as the water solubility of Tween 20 and Tween 80, prompted the investigation of these substances as solubilizers for hexachlorophene. Although Tween 20 and Tween 80 are about equally effective in solubilizing hexachlorophene, Tween 80 was chosen for further investigation because of its official status.

The 1:200, 1:1,000, and 1:2,000 concentrations of hexachlorophene in aqueous Tween solution showed no signs of deterioration when stored in the refrigerator. However, solutions stored at room temperature and those stored at 37° soon developed a mold growth, and upon further standing assumed a milky appearance. This may have been due to a precipitation of hexachlorophene caused by the decomposition of the solubilizer by the mold. The growth of mold in the presence of hexachlorophene is not surprising. It is a known fact that there is often little relationship between the fungicidal and bactericidal activities of a compound.

The presence of a surface-active agent in the hexachlorophene solution suggested use of the total

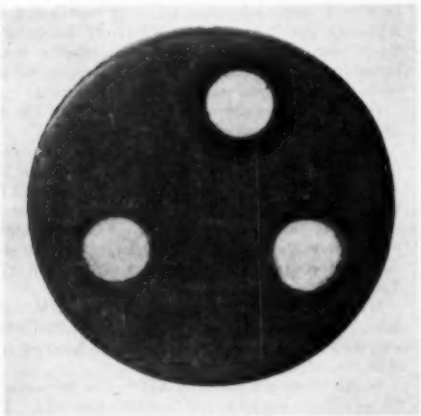

Fig. 1.—Agar cup-plate tests of hydrophilic ointment with hexachlorophene and controls. Left: hydrophilic ointment with Tween 80. Center: hydrophilic ointment with Tween 80 and hexachlorophene. Right: hydrophilic ointment.

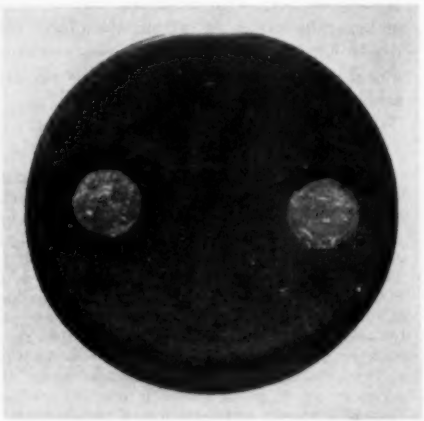

Fig. 3.—White ointment with hexachlorophene and control. Left: white ointment with hexachlorophene. Right: white ointment.

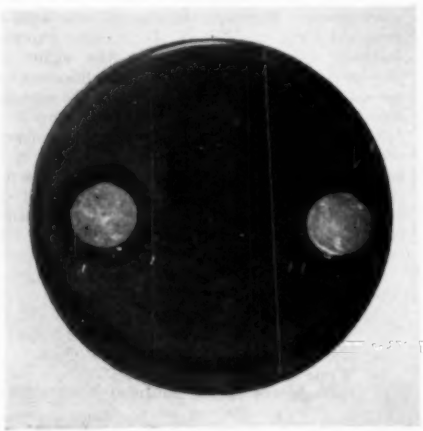

Fig. 2.—Hydrophilic petrolatum with hexachlorophene and control. Left: hydrophilic petrolatum with hexachlorophene. Right: hydrophilic petrolatum.

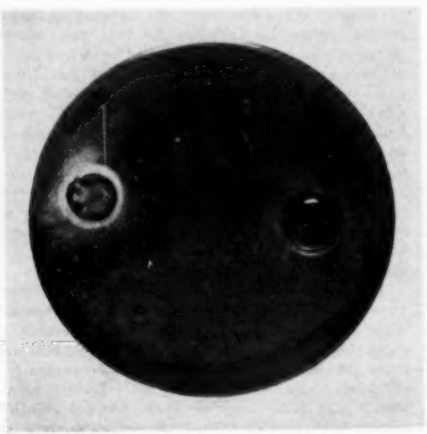

Fig. 4.—Polyethylene glycol ointment with hexachlorophene and control. Left: polyethylene glycol ointment with hexachlorophene. Right: polyethylene glycol ointment.

TABLE I.—AVERAGE ZONES OF INHIBITION PRODUCED BY EACH PREPARATION

Ointment	Width of Zone, Mm.
Hydrophilic ointment with Tween 80[a]	2.0
Hydrophilic ointment with Tween 80 and hexachlorophene	5.5
Hydrophilic ointment[a]	3.0
Hydrophilic petrolatum with hexachlorophene	4.5
Hydrophilic petrolatum[a]	0.0
White ointment with hexachlorophene	5.5
White ointment[a]	0.0
Polyethylene glycol ointment with hexachlorophene[b]	
Polyethylene glycol ointment[b]	

[a] Control.
[b] Polyethylene glycol ointment is soluble in the aqueous medium.

swirl method for germicidal evaluation in preference to the F. D. A. method. The latter has often proved unreliable in the presence of a surface-active agent. The lowered surface tension is thought to interfere with the transference of an aliquot portion with the wire loop. The total swirl method overcomes this factor by the addition of the broth to the culture-medication mixture.

In the time allotted for this study, the phenol coefficient of hexachlorophene, when dissolved in aqueous Tween 80 solution, was not definitely established. It appears that the coefficient will prove to be several times greater than the 125 reported by Gump for the alkali salt.

The discovery of an apparent increase in the bactericidal potency of hexachlorophene in the presence of Tween 80 agrees with the findings of Berthet (9). He found that a surface-active agent, Aerosol OT,[8] in a concentration of 1:2,000 increased the phenol coefficient of hexachlorophene fourfold. Berthet presents three hypotheses as possible explanations of this phenomenon. They are:

(*a*) Chemical combination between the wetting agent and the antiseptic, forming a more active product.

(*b*) If it is accepted that the wetting agent disturbs the cellular membrane, it does so in such a manner as to facilitate the penetration of toxic substances.

(*c*) The wetting agent promotes the concentration of the antiseptic on the bacterial cell body.

There were no significant differences in the widths of the zones of inhibition produced by hexachlorophene, whether incorporated with hydrophilic ointment, hydrophilic petrolatum, or white ointment. Subsequent tests were performed using hydrophilic ointment in which the parabens had been omitted. This base produced no zone of inhibition. It can be assumed that these preservatives are responsible for the zone of inhibition noted when the ointment was tested without hexachlorophene.

Large zones of inhibition, as shown in Fig. 4, were produced by polyethylene glycol ointment and polyethylene glycol ointment with hexachlorophene. This base is completely water soluble; hence, it presents a problem in antiseptic testing. While it is not doubted that hexachlorophene is effective in this base, it is evident that dissolution of the base in the agar medium prevents a reliable comparison with insoluble bases.

Subcultures were made from the zones produced by hexachlorophene in hydrophilic ointment hydrophilic petrolatum, and white ointment. All cultures were negative, indicating the action of hexachlorophene in these bases to be bactericidal in nature rather than bacteriostatic.

It is hoped that this study will be a contridution to the knowledge of the use of hexachlorophene in dermatological pharmaceuticals. It is believed that an aqueous solution of Tween 80 and hexachlorophene would be adaptable to many lotions. Refrigeration or a preservative would be required to prevent mold. Hexachlorophene was successfully incorporated into four official ointment bases. Although the fixed oil solutions of hexachlorophene were not evaluated as to their bactericidal powers, it appears feasible that they will be of use in antiseptic oils.

[8] Trade mark of American Cyanamid Co. for dioctyl sodium sulfosuccinate.

SUMMARY

Hexachlorophene was found to be soluble in aqueous solutions of Tween 20 and Tween 80.

Aqueous Tween-hexachlorophene solutions developed mold growth upon standing. Stock solutions should be refrigerated.

Tests indicated that Tween 80 greatly enhances the bactericidal power of hexachlorophene.

The solubility of hexachlorophene in some common ingredients of ointment bases was determined.

Hexachlorophene was incorporated into four official ointment bases. Modified F. D. A. agar cup-plate tests showed no significant difference in the bactericidal effects of hexachlorophene in hydrophilic ointment, hydrophilic petrolatum, or white ointment. The test proved unsuitable for polyethylene glycol ointment.

Hexachlorophene was shown to be soluble in three vegetable oils. The compound may be solubilized in liquid petrolatum with the aid of Span 85.

It is suggested that hexachlorophene will be adaptable to lotions, ointments, and antiseptic oils.

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Effects of Mercurial Diuretics on Urine Volume and Chloride Excretion in the Rat with Additional Observations on Toxicity*

By H. MINATOYA and JAMES O. HOPPE†

The effects of therargidone, a mercurial diuretic containing 88 mg. of sodium N-(3-hydroxymercuri-2-methoxypropyl)-2-pyridone-5-carboxylate (equivalent to 39 mg. of mercury) and 60 mg. of theophylline per cc., on urine volume and chloride excretion were compared with those of mersalyl and theophylline in the rat. The activity of therargidone was found to be approximately equal to that of mersalyl and theophylline. In acute toxicity determinations, therargidone was found to be more toxic than mersalyl and theophylline in the mouse, equally toxic in the rat, and slightly less toxic in the hamster at twenty-four hours after injection. No delayed deaths of significance were observed with either compound in the mouse. Approximately a fourfold increase in toxicity due to delayed deaths was observed with both substances in the rat and hamster. In subacute toxicity studies in the dog in which dosages of therargidone equivalent to 2, 4, and 8 mg. Hg/Kg. were given subcutaneously once daily, five days a week for six weeks, no evidence of toxicity was observed at the two lower dose levels. An acute nephrosis developed in the dogs at the 8 mg. Hg/Kg. dose level, terminating in death during the second week of medication.

THE LIPSCHITZ, *et al.*, procedure for the bioassay of mercurial diuretics is based on the amount of urine excreted by rats over a period of approximately twenty-four hours (1), and has been used with success by other investigators (2, 3). By definition, an experimental procedure for the estimation of diuretic activity must be capable of measuring any increase in the formation of urine. However, since mercurial diuretics promote mobilization of both salt and water from the tissues, it would seem necessary to investigate not only changes in urine volume but also electrolyte excretion. It is well known that the mercurials cause a pronounced increase in chloride excretion (4–12).

Data in the dog indicate that with chloride excretion as a criterion, it is possible to demonstrate a positive response to mercurial medication in as short a time as one to two hours. Fulton, *et al.* (13), observed an increase in chloride excretion in dogs amounting to 40 to 50 times the control excretion with only a 1.2-fold increase in urine volume within a six-hour experimental period following the intravenous injection of mersalyl. Roby and Pfeiffer (14) obtained a marked increase in chloride excretion in dogs in one to two hours following the intravenous injection of mercurial diuretics. The latter investigators were of the opinion that, if urinary volume alone was used as a criterion of diuresis, many effective therapeutic responses would remain undetected.

There appears to be a dearth of information on the effect of mercurial diuretics on chloride excretion in the rat. The present communication, therefore, is concerned with a comparative study of the effects of therargidone,[1] and mersalyl and theophylline on both urine volume and urinary chloride excretion in the rat, with additional toxicological observations in mice, rats, hamsters, and dogs. Chemically, therargidone is identical with PT-44 with theophylline, as first described by Hunt, Walter, and Fosbinder (15) and HMP as reported by Cahen (2). The material used in this study differs from these only in respect to the proportions of the mercurated organic moiety and theophylline as identified under Methods.

METHODS

The following ampulled solutions were used either undiluted, or when necessary, diluted with distilled water in this investigation: mersalyl and theophylline, containing 100 mg. of mersalyl (equivalent to 39.6 mg. of mercury) and 50 mg. of theophylline per cc.; and therargidone, containing 88 mg. of sodium N-(3-hydroxymercuri-2-methoxypropyl)-2-pyridone-5-carboxylate (equivalent to 39 mg. of mercury) and 60 mg. of theophylline per cc.

Diuretic Activity

The effects on both urine volume and urinary chloride excretion were determined following intramuscular as well as subcutaneous injection in rats. The procedure employed for the estimation of urinary chlorides was essentially that of Schales and Schales (16).[2]

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† The authors are very grateful to Dr. F. C. Goble for the gross and microscopic tissue studies, and to Mr. D. K. Seppelin for technical assistance.

[1] This preparation has been assigned the trade name Merdroxone by Winthrop-Stearns Inc., and the trade name Mergamate by Maltbie Laboratories, Inc.

[2] The authors are very grateful to Mr. M. E. Auerbach of this Institute for technical advice on the chloride determinations.

The dose-response relationships for both substances were obtained by administering dosages equivalent to 2, 4, 8, and 16 mg. Hg/Kg. Thirty or more healthy female rats weighing 150–220 Gm. were fasted without water overnight (sixteen to eighteen hours) prior to each experiment. In the morning each rat was given 2.5 cc. of isotonic sodium chloride solution per 100 Gm. of body weight by stomach tube. The rats were then placed three to an individual metabolism cage for urine collection. One hour after the saline was given, the amount of urine excreted was recorded and set aside for chloride estimation. Immediately following the one-hour control collection period, two groups of six rats each were injected either intramuscularly or subcutaneously at two different dose levels with therargidone, and two groups with the same dosages of mersalyl and theophylline in a volume of approximately 0.2 cc. A fifth group was injected with a similar volume of distilled water as a control.

Urine samples were collected at one hour after medication and at one and one-half hour intervals thereafter for a total of six and one-half hours from the time the normal saline was given. Any residual urine in the bladder was expressed by moderate compression on the lower abdomen at each collection period. The total chloride excretion expressed in mg. of sodium chloride was obtained from the product of urinary volume and the mg. of sodium chloride per cc.

Toxicity

Acute Toxicity.—The acute toxicity following single doses of therargidone was determined in comparison with mersalyl and theophylline by intravenous injection in mice and hamsters and by subcutaneous injection in mice and rats. The intravenous doses were administered at a rate of 1.0 cc. per minute and the volume of all injections was 0.35 ± 0.15 cc. The LD_{50} ± its standard error was estimated both at the end of twenty-four hours and seven days by the method of Miller and Tainter (17).

Subacute Toxicity in Dogs.—Therargidone was administered undiluted by subcutaneous injection once daily, five days a week to adult, healthy female mongrel dogs as follows: 2 mg. Hg/Kg., two dogs for six weeks; 4 mg. Hg/Kg., one dog for six weeks and one dog for three weeks (discontinued because of pregnancy); 8 mg. Hg/Kg., two dogs for approximately two weeks when it was necessary to terminate these injections because of severe toxic manifestations. The dogs were weighed once a week and periodic blood nonprotein nitrogen and urine studies were made throughout the test. Gross and microscopic tissue examinations were made either during or at the termination of the experiment as noted later.

RESULTS

Diuretic Activity in Rats

A maximal increase in urine volume and urinary chloride excretion was obtained with both mercurials approximately one hour after intramuscular injection, as illustrated by a typical response at 8 mg. Hg/Kg. in Fig. 1. In marked contrast, urine and chloride excretion in the control group, which received a sham injection of distilled water, remained

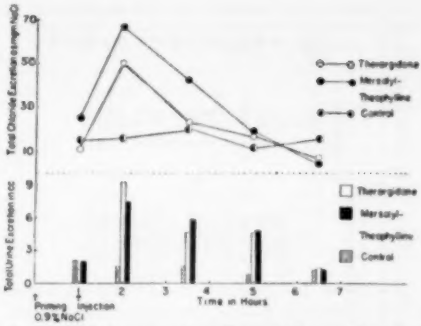

Fig. 1.—The comparative effects of therargidone and mersalyl and theophylline at 8 mg. Hg/Kg. by intramuscular injection in rats (average of six rats each).

relatively constant throughout the six and one-half hour experimental period. This time-effect relationship quite closely resembles the results obtained with mercurial diuretics in dogs by Fulton, *et al.* (13). It will also be noted in Fig. 1 that both urine and chloride excretion decreased after five hours to a rate somewhat less than that at the beginning of the experiment.

Although there was some increase in urine volume and urinary chloride excretion following the intramuscular injection of both therargidone and mersalyl and theophylline at a dosage equivalent to 2 mg. Hg/Kg., the increase was not significantly different from that of the controls as shown in Table I. However, significant increases were obtained with both drugs at doses equivalent to 4 and 8 mg. Hg/Kg. At the 16 mg. Hg/Kg. dose level, urine volume was diminished to a point approximately equal to that of the controls, but the chloride excretion was significantly decreased below that of the controls. Lipschitz, *et al.* (1), also observed that overdosage with a mercurial diuretic produced a definite decrease in urinary excretion. Roby and Pfeiffer (14) found a marked decrease in chloride excretion at doses greater than 6 mg. Hg/Kg. in their studies on mercurial diuretics in dogs.

There was no evidence of a significant increase in chloride excretion when therargidone was given by subcutaneous injection. However, a significant increase in urine volume was obtained at the 8 mg. Hg/Kg. dose level. It will be noted in Table I that a significant decrease in chloride excretion also occurred at 16 mg. Hg/Kg. by this route of administration.

A composite comparison of the dose-response and dose-mortality curves for both substances in rats is shown in Fig. 2. It will be seen that, following the intramuscular injection of therargidone, chloride excretion increased up to the 4 mg. Hg/Kg. dose level, after which there was a decline associated with an increase in mortality. Urinary volume, however, continued to increase in spite of the increase in mortality up to the 8 mg. Hg/Kg. dose level. At the 16 mg. Hg/Kg. dose, both urine volume and chloride excretion had decreased to a point equal to or below that of the controls, while the mortality rate exceeded 90%. In the case of mersalyl and theophylline, there was a slight increase in chloride excretion

TABLE I.—THE DIURETIC EFFECT OF SINGLE DOSES OF THERARGIDONE AND MERSALYL-THEOPHYLLINE OVER A SIX AND ONE-HALF HOUR TEST PERIOD IN RATS

Compound	Dose, Mg. Hg/Kg.	Route of Adm.	No. of Groups (6 Rats per Group)	Cc. of 0.9% Saline Given (Mean)	Mean Diuretic Activity — Total Urine Excretion — Cc. ± S. E.	P	Relative Activity Controls = 1.00	Total Chloride Excretion — Expressed as Mg. NaCl ± S. E.	P	Relative Activity Controls = 1.00	Mortality, %
Therargidone	2	i.m.	4	26.7	6.6 ± 2.2	0.6	1.22	81.2 ± 18.3	0.94	1.02	0
	4		4	27.8	16.5 ± 2.1	<0.01	3.06	145.2 ± 15.5	<0.01	1.85	8
	8		4	27.3	19.9 ± 0.6	<0.01	3.7	113.6 ± 7.6	<0.01	1.43	46
	16		4	27.2	4.9 ± 2.1	0.8	0.91	39.8 ± 8.7	<0.01[a]	0.51	92
	2	s.c.	4	24.8	5.9 ± 0.4	0.7	0.97	84.3 ± 3.3	0.6	1.04	0
	4		4	24.1	8.2 ± 1.9	0.3	1.34	91.3 ± 16.6	0.6	1.13	0
	8		4	24.4	15.4 ± 2	<0.01	2.53	96.4 ± 14.3	0.3	1.19	21
	16		4	24.1	6.7 ± 0.9	0.6	1.1	40.0 ± 2.3	<0.01[a]	0.49	92
Mersalyl-theophylline	2	i.m.	4	28.7	7.0 ± 1.7	0.4	1.29	94.5 ± 17.2	0.4	1.19	0
	4		4	29.0	12.6 ± 1.4	<0.01	2.34	128.0 ± 5.7	<0.01	1.61	0
	8		4	28.3	20.0 ± 0.1	<0.01	3.7	139.1 ± 7.7	<0.01	1.75	4
	16		4	27.2	5.4 ± 1.7	1.0	1.0	31.6 ± 6.2	<0.01[a]	0.39	92
Control	..	i.m.	12	27.6	5.4 ± 0.4	...	...	79.6 ± 6.5	...	...	0
	..	s.c.	4	23.4	6.1 ± 0.4	...	...	80.8 ± 6.4	...	...	0

[a] Significant decrease.

Fig. 2.—Diuretic activity in rats. Combined comparative dose-response and dose-mortality curves for therargidone and mersalyl and theophylline by intramuscular injection in rats (24 rats/dose level).

after the 4 mg. Hg/Kg. dose, followed by a sharp decrease after the 8 mg. Hg/Kg. dose level when the mortality rate assumed an abrupt increase. Urinary excretion continued to increase at a fairly rapid rate between the 4 and 8 mg. Hg/Kg. dose levels, whereas the increase in chloride excretion had become much slower after the 4 mg. Hg/Kg. dose level. As with therargidone, both urine and chloride excretion had almost ceased at 16 mg. Hg/Kg., and the mortality rate reached 92%. The deaths occurred over a period of one to five days following medication. Albuminuria was observed with both mercurials at the high dose levels.

Acute Toxicity.—As seen in Table II, therargidone was found to be more toxic than mersalyl and theophylline in the mouse; equally toxic in the hamster at twenty-four hours after injection. There were no delayed deaths of significance with either compound in the mouse. The relative toxicity of therargidone and mersalyl and theophylline in the mouse was found to be similar to that reported by Hunt, *et al.* (15). There was approximately a four-fold increase in toxicity of both preparations due to delayed deaths in the rat and hamster. It is interesting to note that the toxicity of the compounds was approximately the same at seven days after injection in rats and hamsters.

When administered at high dose levels by intravenous injection, both preparations produced terminal tonoclonic convulsions and death within three to five minutes in mice and hamsters. The deaths appeared to be a result of respiratory failure, although infrequently cardiac arrest was observed to precede respiratory failure in mice. At dosages which were not immediately fatal, the animals became depressed and lapsed into a semicomatose condition followed by death in a few hours after injection. No delayed toxic manifestations were observed in mice but the hamsters became emaciated and died in one to five days after intravenous injection.

There were no apparent immediate effects following subcutaneous administration. The early deaths occurred at approximately an hour after medication. There was only an occasional delayed death among the mice, but the rats became emaciated and died in from three to five days following medication.

Subacute Toxicity in Dogs.—There were no obvious changes in body weight or symptoms of systemic intoxication in any of the four dogs at the 2 and 4 mg. Hg/Kg. dosage levels. Qualitative tests for albumin in the urine were negative, and microscopic

TABLE II.—COMPARISON OF THE ACUTE TOXICITY OF THERARGIDONE AND MERSALYL-THEOPHYLLINE

Compound	Species	No. of Animals	Route of Adm.	LD_{50} ± S. E. Expressed in Mg. Hg/Kg. At 24 Hr.	After 7 Days
Therargidone	Mouse	50	i.v.	15 ± 1	15 ± 1
	Hamster	57	i.v.	44 ± 3	10 ± 1
	Mouse	30	s.c.	66 ± 9	53 ± 3
	Rat	21	s.c.	44	15
Mersalyl-theophylline	Mouse	50	i.v.	45 ± 4	45 ± 4
	Hamster	72	i.v.	36 ± 3	11 ± 2
	Mouse	40	s.c.	95 ± 16	87 ± 16
	Rat	21	s.c.	40	14

examination of centrifuged urine samples from these dogs revealed no significant changes during the course of the experiment. Periodic blood nonprotein nitrogen determinations revealed no significant changes.[8] No evidence of irritation was observed as a result of the subcutaneous injection of therargidone at these two dose levels.

Both dogs at the 8 mg. Hg/Kg. dose level developed early severe symptoms of mercurial intoxication. Within two to three days after the start of medication, the urine of both dogs showed the presence of excessive amounts of albumin. The urine also contained granular casts and both red and white cells. Urine excretion rapidly diminished in one dog after the third day, followed by a marked loss in body weight, eventual anuria, and a generalized state of acute inanition. A similar pattern of events developed in the second dog at this dose level at the end of the first week. Terminal blood nonprotein nitrogen values in excess of 100 mg. % were found in each case. Both dogs became moribund and were sacrified during the second week of the experiment.

Autopsy data at the 8 mg. Hg/Kg. dosage level demonstrated focal extravasation of blood in the myocardium with resulting hemosiderosis and leukocytic infiltration. The kidneys exhibited acute nephrosis. The damage was extensive and diffuse but it could be observed that the proximal convoluted tubules were most severely affected and the glomeruli were the least involved. No gross or microscopic tissue changes attributable to the medication were observed in the two dogs which had received 2 mg. Hg/Kg. and in one dog at 4 mg. Hg/Kg. Medication of the second dog at this dose level had been discontinued after the third week because of pregnancy. This dog later delivered a normal litter of pups. Neither the mother nor the pups showed any immediate or delayed effects during or following the nursing period as a result of the medication.

DISCUSSION

Cahen (2) found the diuretic activity of therargidone to be about the same as that of mersalyl and theophylline in the rat. In the present experiments, the activity of therargidone by intramuscular injection in the rat, as judged by urine and urinary chloride excretion, was also found to be similar to that of mersalyl and theophylline, in agreement with Cahen's findings. It is interesting to note, however, that evidence of activity was obtained within a six and one-half hour observation period, compared to the twenty-four hour urine collection period used by Cahen. In fact, the maximum increase in both urine and urinary chloride excretion occurred at approximately one hour after injection of the mercurials.

The maximum increase in urine output was found to be 370%, whereas the maximum increase in chloride excretion amounted to 185%, or less in the rat. As seen in Table I, greater increases were demonstrated when urinary volume was used to express diuretic activity. However, a rise in the mortality rate occurred at a point where the rate of chloride excretion began to diminish, while urine volume was still increasing (Fig. 2). It would appear that chloride excretion may be a better criterion of therapeutically useful degrees of mercurial diuresis. The chloride excretion and mortality curves in Fig. 2 seem to indicate that the point at which a downward trend occurs in chloride excretion marks the change from maximum tubular inhibition to the beginning of irreversible tubular and glomerular damage, as suggested by Roby and Pfeiffer (14).

The present study indicates that the rat serves as a convenient test animal for routine studies on mercurial diuretics, although the data obtained on chloride excretion in the rat are far less dramatic than the data obtained by Fulton, *et al.* (13), in the dog. The magnitude and variation of the response in the rat makes it imperative that a sufficient number of animals be used for each test group and that each experiment be made in comparison with a control group. It is usually better, as pointed out by Lipschitz, *et al.* (1), to perform at least three or four such controlled experiments so as to average day to day variations. The results may also be put on a more nearly comparable basis if the diuresis is expressed as a ratio to the effects in the sham injected control group.

The greatest difficulty inherent in nearly all the procedures described for testing diuretics lies in the fact that the tests are made on normal animals. It is unreasonable to expect the tissues of a normal animal to surrender tissue fluid in response to medication with a diuretic compound in the same proportion as in an edematous patient. It becomes necessary, therefore, to use a priming fluid in order to demonstrate any diuretic response at all. The dose of 25 cc. of physiological saline per kilogram of body weight, as recommended by Lipschitz, *et al.* (1), has been found to be satisfactory as a priming dose in the rat. When this same dose of tap water was used, a pronounced increase in urine containing a very low concentration of chloride was obtained. As seen in Fig. 1, this dose of saline produced no significant change in either urine or chloride excretion during the experimental period. Preliminary

[8] The authors are grateful to Mr. John McAuliff of this Institute for the blood N. P. N. determinations.

experiments have shown that this dose is not an excessive amount of fluid since rats which had been fasted without water overnight were observed to drink of their own volition from 20 to 40 cc./Kg. of physiological saline over and above the 25 cc./Kg. administered as a priming dose.

SUMMARY

1. The diuretic activity of therargidone by intramuscular injection in rats appears to be approximately the same as that of mersalyl and theophylline.

2. A procedure for the estimation of the effects of these two mercurial diuretics in rats by means of the increases in urine and chloride excretion over a six and one-half hour test period has been described.

3. Both mercurials produced a maximum increase in urine volume of 370 per cent, whereas the increase in chloride excretion amounted to 185 per cent or less.

4. Chloride excretion was observed to decrease at the higher dose levels. The rat studies indicated that an increase in mortality occurred as soon as the rate of chloride excretion began to diminish, while urinary excretion continued to increase in spite of an increase in mortality rate.

5. No evidence of either acute or cumulative toxicity was observed during the daily administration of therargidone at dosages equivalent to 2 and 4 mg. Hg/Kg. in dogs. No gross evidence of irritation at the sites of injection was observed.

6. Doses of therargidone equivalent to 8 mg. Hg/Kg. were found to be cumulative, resulting in death of the test dogs within two weeks. The histopathological findings on representative tissues have been presented.

7. In acute studies, therargidone was observed to be more toxic than mersalyl and theophylline in the mouse, equally toxic in the rat, and slightly less toxic in the hamster at twenty-four hours after medication. No delayed deaths of significance were observed with either compound in the mouse. Approximately a fourfold increase in toxicity due to delayed deaths was observed with both substances in the rat and hamster.

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WHO MAKES IT?

The National Registry of Rare Chemicals, Armour Research Foundation, 33rd, Federal and Dearborn Streets, Chicago, Ill., seeks information on sources of supply for the following chemicals:

2,3,4-Trimethylhydroxybenzene
2,4,6-Trimethylhydroxybenzene
Methyl cellobioside
Ovalene
Ovomucin
Parinarum laurinum
Phloretin
Phosphoenol pyruvic acid
Rennin
Sodium cholesteryl phthalate
γ-Terpineol
Methyl erucate
Aleuronate
Copper thiocholine
Gymnemic acid
Liquiritin
Benznaphthacene
4′-Ethoxy-2,4-diamino-5-methylazobenzene

Reduction of Alloxan in Aqueous Solution. I. By Hydrogen Sulfide*

By R. STUART TIPSON and LEONARD H. CRETCHER†

Study of the reduction of alloxan in aqueous solution with hydrogen sulfide reveals that, in deciding the products formed (at 25° or the boiling temperature), temperature is not a factor (except in so far as decomposition may be accelerated by rise in temperature). It is possible, by appropriate choice of the proportion of hydrogen sulfide introduced, to produce at will alloxantin dihydrate or dialuric acid monohydrate, regardless of the temperature or the initial alloxan concentration.

IN CONNECTION with a study of the causation of experimental diabetes in animals by administration of aqueous alloxan solution, we have examined the effect of various reductants on alloxan in aqueous solution, and have encountered behavior not in precise concordance with predictions based on the information at present available in the literature. We have therefore reinvestigated the conditions under which alloxantin and dialuric acid are formed when hydrogen sulfide is the reductant.

EARLY OBSERVATIONS

In 1838, Wöhler and Liebig (1) found that, on passing some hydrogen sulfide into a concentrated aqueous solution of alloxan *at room temperature*, sulfur was precipitated and colorless crystals also separated (Experiment 1). This mixed precipitate was filtered off, treated with boiling water (in which the colorless crystals dissolved), and the sulfur was filtered off. On cooling the filtrate, colorless crystals of a material they called "alloxantin" were obtained. Elementary analysis of this material indicated the formula $C_8H_{10}N_4O_{10}$, or, more precisely (2–4), $C_8H_6N_4O_8 \cdot 2H_2O$.

On the other hand, they observed that, on passing *excess* hydrogen sulfide into a *boiling* aqueous solution of this alloxantin, "eine neue Veränderung" occurred (Experiment 2). A further quantity of sulfur was precipitated, possibly implying that hydrogen sulfide has a more potent reducing action in the boiling reaction mixture than at room temperature. The organic product, which they named "dialuric acid," could not be isolated free (by them), but they prepared its ammonium salt; this had an elementary analysis indicating the formula $C_8H_{14}N_6O_8$ (modern, $C_4H_7N_2O_4$). From this they deduced that dialuric acid has the formula $C_8H_8N_4O_8$ (modern, $C_4H_4N_2O_4$), and suggested that alloxantin and dialuric acid might be compounds of alloxan with hydrogen. The same ammonium salt could be isolated by treating a boiling *alloxan* solution with excess hydrogen sulfide and then with ammonium carbonate (Experiment 3). [A few years later, Gregory (5) succeeded in isolating dialuric acid monohydrate by the action of dilute hydrochloric acid on the ammonium salt.]

Wöhler and Liebig (1) decided that alloxantin represents an intermediate stage of reduction, between alloxan and dialuric acid, for the following reason (Experiment 4). They passed hydrogen sulfide into a *boiling* aqueous solution of alloxan until reduction was judged to be complete; the solution was sufficiently dilute that, on cooling, organic product did not crystallize out. Addition of some alloxan solution to the filtrate now caused crystallization of some alloxantin. [They were puzzled, however, as to how to reconcile the above results with their observation that, if an alloxan solution was treated with some hydrogen sulfide *in the cold* and the mixed precipitate was filtered off, addition of some alloxan solution to the filtrate gave a second crop of alloxantin, suggesting that the mother liquor had contained dialuric acid. This observation was confirmed by their pupil, Schlieper (6).]

Subsequent authors misquoted Wöhler and Liebig's Experiment 4 and implied that 1 mole of alloxan was added to 1 mole of dialuric acid to yield 1 mole of alloxantin. Thus, with no additional experimental proof, Strecker (7) depicted alloxantin as a 1:1 compound of alloxan with dialuric acid, and Baeyer (8), also without further proof, utilized this formulation. One of Baeyer's equations, modernized, is as follows:

$$\underset{\text{Alloxan monohydrate}}{C_4H_2N_2O_4 \cdot H_2O} + \underset{\text{Dialuric acid monohydrate}}{C_4H_4N_2O_4 \cdot H_2O} = \underset{\text{Alloxantin dihydrate}}{C_8H_6N_4O_8 \cdot 2H_2O} \quad (1)$$

This interpretation of Wöhler and Liebig's experimental observations does not appear to have been verified (9) until 1913 (and, even then, not very precisely).

PRESENT WORK

It appeared that one of the simplest ways of testing these hypotheses would be to repeat Wöhler and Liebig's experiments, first determining how much sulfur is liberated on passing excess hydrogen sulfide into cold, and into boiling, aqueous solutions of given weights of alloxan monohydrate. According to Baeyer's theory, the equations (modernized) for these reactions should be:

$$2C_4H_2N_2O_4 \cdot H_2O + H_2S = C_8H_6N_4O_8 \cdot 2H_2O + S \quad (2)$$

$$C_4H_2N_2O_4 \cdot H_2O + H_2S = C_4H_4N_2O_4 \cdot H_2O + S \quad (3)$$

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We soon learned that, in at least one respect, the behavior observed at room temperature depended on the initial concentration of alloxan monohydrate; if this was at or below a certain critical value (x Gm. per liter), the precipitate finally deposited consisted solely of sulfur; above this concentration, an organic compound also crystallized out.

It was found that, in partial agreement with Wöhler and Liebig's observations (1), treatment of aqueous alloxan solution with excess hydrogen sulfide, first at room temperature and then *at the boiling temperature* of the mixture, proceeds in accordance with Eq. 3. Surprisingly, however, *in the cold* (25°) also, treatment of a solution of less than the "critical" concentration with excess hydrogen sulfide causes the liberation of the amount of sulfur demanded by Eq. 3 (instead of Eq. 2); there was no immediate evidence for a halting of the reaction at the "half-way" stage represented by Eq. 2. [However, the observation that, in the zone of alloxan concentrations above a certain figure, z (but below the "critical" concentration x), some colorless crystals formed and then redissolved, during the treatment with hydrogen sulfide, suggested that the reaction might have proceeded via that pathway.]

After filtering off the sulfur produced in the reaction at 25°, a good yield of colorless, crystalline organic product (X) was readily isolated by cooling the filtrate in ice, provided that the initial alloxan concentration had been at or only slightly below x. We now proceeded to determine the effect of excess hydrogen sulfide on cold and boiling solutions of this supposed "alloxantin dihydrate." According to Baeyer's theory, there should be no reaction at room temperature, but, in the boiling solution, reaction should occur as in Eq. 4.

$$C_8H_4N_4O_8 \cdot 2H_2O + H_2S = 2C_4H_4N_2O_6 \cdot H_2O + S \quad (4)$$

It was found that if X was dissolved in cold deaerated water, and excess hydrogen sulfide was passed in, no sulfur was liberated; this is not at variance with expectations. But when the same treatment was performed at the boiling temperature of the solution, there was *also* no liberation of sulfur. Hence, compound X was dialuric acid monohydrate and, under the above conditions, Eq. 4 represents, as far as the over-all effect and final result is concerned, a possibly nonexistent reaction. To summarize, the only reaction for which we found positive evidence (at this stage in the investigation) is that shown in Eq. 3; in other words, treatment of alloxan solution (of concentration less than x) with *excess* hydrogen sulfide yields *dialuric acid* as the sole final organic product, no matter whether the reaction be performed at 25° or at the boiling temperature.

Tentatively, it therefore seemed that, under the above conditions, alloxantin may be regarded as nonexistent—a conclusion in partial conformity with Piloty's statement (10), confirmed by later workers (11–16), that an aqueous solution of alloxantin behaves as a solution of alloxan plus dialuric acid. Indeed, Hill and Michaelis (17) had come to the conclusion that "the intermediary form, known as alloxantin in the crystalline state, does not exist to any appreciable extent" in solution.

Consequently, it seemed necessary to repeat Wöhler and Liebig's Experiment 4 on the formation of alloxantin. On adding a deaerated aqueous solution of 1 molar proportion of alloxan monohydrate to a deaerated aqueous solution of 1 molar proportion of dialuric acid (prepared as above, from an alloxan solution of the "critical" concentration), an excellent yield of crystalline alloxantin dihydrate was deposited (Eq. 1), thus reconfirming Wöhler and Liebig's result and Baeyer's formulation.

Being now possessed of authentic alloxantin dihydrate, we proceeded to study the effect of an excess of hydrogen sulfide on cold and hot aqueous solutions of given weights of the compound. Treatment of a saturated or undersaturated, deaerated, aqueous solution at room temperature *proceeded according to Eq. 4*, with no deposition of organic product. (When a cold, aqueous *suspension* was so treated, all the alloxantin dihydrate dissolved and no organic crystals separated if the proportion of alloxantin dihydrate to water was less than that corresponding to x. Hence z represents the alloxan monohydrate concentration giving a saturated solution of alloxantin composition.) Reaction near the boiling temperature also followed Eq. 4. Because dialuric acid is more soluble than alloxantin in both cold and hot water, the result is independent of the initial alloxantin concentration.

On the other hand, as regards the reduction of alloxan solutions, the "critical" concentration x is obviously determined by the weight of alloxan monohydrate which will give a saturated solution of dialuric acid in the presence of excess hydrogen sulfide (and crystal nuclei of dialuric acid monohydrate). In the absence of these nuclei, there appears in respect to dialuric acid a supersolubility zone, the lower limit of which is defined by x. Although it affects the solubilities, in the reaction mixture, of the various compounds involved, the *temperature* at which the reaction is performed does not influence the results (except in so far as decomposition may occur near the boiling temperature). Instead, the real controlling factor, hitherto apparently unrecognized, is the proportion of hydrogen sulfide introduced. It was found that, for alloxan solutions *below the "critical" concentration*, addition at 25° of exactly 1 mole of hydrogen sulfide per 2 moles of alloxan proceeded according to Eq. 2. Alloxantin dihydrate crystallized out if the resulting aqueous solution was supersaturated therewith.

Exactly the same kind of result was obtained with alloxan solutions *above the "critical" concentration*. That is to say, using excess hydrogen sulfide, *all* the alloxantin was reduced to dialuric acid in accordance with Eq. 4 (over-all, Eq. 3). The "critical" concentration has no significance in this respect.

To summarize, alloxantin dihydrate cannot be prepared by the action of excess hydrogen sulfide on aqueous alloxan solutions under any conditions studied, despite the numerous reports to this effect in the literature. It is thus evident that, in the preparation of alloxantin dihydrate (1, 5, 18–21), either the use of *excess* hydrogen sulfide is to be avoided (because of the resulting complete transformation to dialuric acid), or sufficient concentrated alloxan solution should be subsequently added to permit recovery as crystalline alloxantin dihydrate. [The latter method was employed by Schlieper (6).]

In the same connection, although dialuric acid monohydrate has been prepared many times in the past, it is doubtful if it has often been *isolated* hitherto [*cf.*, Baeyer (8)] in pure form. In preparing

dialuric acid monohydrate, heating is not only unnecessary but may be deleterious; furthermore, operation at room temperature is simpler.

DISCUSSION

It should be noted that, in describing their preparation (Experiment 1) of alloxantin dihydrate from alloxan monohydrate, Wöhler and Liebig (1) did not stipulate the use of *excess* hydrogen sulfide, but some subsequent authors (5, 6, 19, 21) have definitely advocated the use of excess of this reductant and state that alloxantin dihydrate then crystallized out. Just because the latter is less soluble (in water at room temperature) than is either alloxan monohydrate or dialuric acid monohydrate, it was assumed that the organic precipitate formed on treatment with excess hydrogen sulfide was alloxantin dihydrate. As we have shown, the latter would only be obtained if less than an excess of hydrogen sulfide were deliberately or fortuitously introduced. [But alloxantin dihydrate could readily result as an *artifact* through the subsequent action of atmospheric oxygen on the dialuric acid monohydrate during filtration, drying, or recrystallization.] The same reasoning probably also applies to such reductions as that of ninhydrin to hydrindantin (22), where the use of excess hydrogen sulfide has been advocated. Furthermore, despite the claims in the literature, we see no reason for believing that the action of the various other reductants recommended for use instead of hydrogen sulfide differs from that outlined above, if the reducing power of these reductants is comparable to that of hydrogen sulfide. Such reductants include zinc and hydrochloric acid (1, 23), sodium amalgam (8, 9), hydriodic acid (14, 24), tin and hydrochloric acid (8, 13), and stannous chloride (1, 2, 9, 13, 23–29). It should be noted that, in 1930, Biilmann and Mygind (30) proved that use of stannous chloride for the preparation of dimethylalloxantin by Biltz's method (31) actually yields methyldialuric acid.

EXPERIMENTAL

Deaerated Water.—Distilled water was deaerated by adding a boiling stone, boiling under reflux for at least five minutes, and then cooling in ice to room temperature, under an atmosphere of oxygen-free nitrogen.

Moist Hydrogen Sulfide.—Hydrogen sulfide was passed through a reversed, empty 500-ml. Drechsel gas-washing bottle and then through 250 ml. of deaerated water in a similar bottle.

Moist Oxygen-Free Nitrogen.—Nitrogen was passed through: (*a*) a 500-ml. Drechsel bottle containing a solution (prepared fresh each day) of 25 Gm. of sodium hydroxide plus 5 Gm. of pyrogallol in 250 ml. of deaerated water; (*b*) a reversed, empty 500-ml. bottle; and (*c*) a 500-ml. bottle containing 250 ml. of deaerated water. The resulting gas will be referred to as "nitrogen."

Alloxan Monohydrate.—Alloxan monohydrate was dried to constant weight over soda lime and phosphorus pentoxide in the vacuum desiccator at room temperature. It was colorless, and readily and completely soluble in five volumes of cold water. It assayed 99–100% alloxan monohydrate by Tipson and Cretcher's method (32).

Action of Excess Hydrogen Sulfide on Alloxan Monohydrate.—(*a*) *At 25°.*—Distilled water (1 L.) was placed in a tared, 1,500-ml. Erlenmeyer flask and deaerated; 16.01 Gm. (0.1 mole) of alloxan monohydrate was added, and the mixture weighed and then swirled gently until the alloxan had dissolved. Hydrogen sulfide was immediately passed in, with resulting liberation of colloidal sulfur followed by deposition of colorless crystals. When ca. 7 Gm. of hydrogen sulfide had been absorbed, the sulfur readily settled out. The precipitate was now filtered off by suction, while hydrogen sulfide was passed in. [The filtering apparatus (preflushed with hydrogen sulfide) was a simplified macro version of that used in microanalysis (33); a tared Gooch crucible having a coarse, fritted-glass septum was employed.] The crucible plus precipitate was then immediately transferred to a vacuum desiccator (preflushed with hydrogen sulfide) and dried overnight over soda lime and phosphorus pentoxide; wt. of precipitate, 6.8 Gm. The precipitate was suspended in 250 ml. of water, the suspension boiled under reflux during fifteen minutes and filtered hot, and the precipitate redried; wt., 3.2 Gm. (unchanged on further washing and drying).

The solubility of alloxantin dihydrate at room temperature has been variously reported as 8.182 Gm. per liter of water acidulated with hydrochloric acid (4), 2.8 Gm. per liter of aqueous solution (12), 2.886 Gm. per liter of aqueous solution saturated with carbon dioxide (14), and ca. 2.674 Gm. per liter of solution in 0.05 *N* aqueous hydrochloric acid (16). The solubility of dialuric acid monohydrate at room temperature is said to be ca. 4.053 Gm. per liter of solution in 0.05 *N* aqueous hydrochloric acid (16). From our experiment reported above, it is seen that the organic product is soluble to the extent of ca. 12.6 Gm. per liter of water at 25° when the solution is saturated with hydrogen sulfide.

Consequently, in order to obtain a mother liquor containing all the organic product, the experiment was repeated, except that 1,300 ml. of water was used. On passing in hydrogen sulfide, the colorless crystals, which started to form soon after liberation of sulfur commenced, slowly redissolved and, after a time, only sulfur was visible. The weight of this precipitate (dry) was 3.2 Gm. (unchanged on treatment with boiling water as described above). (The main *mother liquor* was now heated and kept at the boil for sixty minutes, under reflux, while hydrogen sulfide was admitted through a tube passing down the middle tube of the condenser. No more sulfur was liberated.)

To permit ready isolation of the organic product formed below the "critical" concentration, the experiment was now repeated in a separatory funnel, except that, after excess hydrogen sulfide had been passed in, 100 ml. of carbon disulfide was added. The opalescence in the aqueous layer disappeared, the carbon disulfide acquired a yellow color, and a trace of insoluble, white material separated at the liquid-liquid interface. The carbon disulfide layer was separated, and evaporated to dryness; wt. of sulfur, 3.2 Gm. The aqueous layer was now cooled to +2° in ice, while hydrogen sulfide was passed in; colorless crystals were deposited. The flask was stoppered and kept overnight in the refrigerator, the mixture was filtered as described above, and the crystals (washed with carbon disulfide and delib-

erately left reeking with carbon disulfide and hydrogen sulfide) dried in the vacuum desiccator (Desiguard) over soda lime and phosphorus pentoxide under high vacuum (with trap cooled in Dry Ice-chloroform); wt. (dry), 9.5 Gm. From its infrared absorption spectrum (34), this material proved to be dialuric acid monohydrate.

For isolation of the organic product crystallizing out at room temperature when the strength of the original alloxan solution was *above the "critical" concentration*, 32.02 Gm. of alloxan monohydrate in 1,273 ml. of water was treated with excess hydrogen sulfide at 25°, yielding 25.5 Gm. of initial precipitate (dry); extraction of the precipitate with cold carbon disulfide gave 6.4 Gm. of sulfur and 19.1 Gm. of colorless crystals insoluble in carbon disulfide. [From its infrared absorption spectrum (34), the latter proved to be dialuric acid monohydrate.] The aqueous filtrate gave no more free sulfur on retreatment with hydrogen sulfide. It was then cooled in ice to 2°, saturated with hydrogen sulfide, and kept overnight in the refrigerator, yielding a further 7.1 Gm. (dry) of colorless crystals of dialuric acid monohydrate.

(*b*) *At the Boiling Temperature.*—The second experiment described above was repeated except that, after the distilled water (1,300 ml.) had been boiled under reflux (Liebig condenser with large bulbs), it was only allowed to cool slightly. Then 16.01 Gm. of alloxan monohydrate was added; some decomposition apparently took place since a gas was evolved. Hydrogen sulfide was immediately passed in (through a tube passing through the center tube of the condenser), giving colloidal sulfur; this gas stream was continued while the suspension was reheated and then boiled for seventy minutes. The sulfur was now present as a yellow suspension which settled readily; it was filtered (hot) while hydrogen sulfide was passed in; wt. (dry) = 0.93 Gm. (unchanged by further washing and drying).

Now, Wöhler and Liebig (35) have demonstrated that, on boiling an aqueous solution of alloxan, carbon dioxide is liberated and a mixture of alloxantin and parabanic acid results. Since this reaction had apparently occurred to some extent in the above experiment, the reaction was repeated with the following modification. After the water had been deaerated, it was cooled in ice to room temperature while hydrogen sulfide was passed in to saturation. The alloxan monohydrate was then added, giving an immediate precipitate of sulfur. The gas stream was continued, while the suspension was reheated and then boiled for five minutes. Since all the free sulfur was now present as a yellow suspension which settled readily, and since no more was liberated, it was filtered from the hot suspension as described above; wt. (dry) = 3.2 Gm. (unchanged by extraction with boiling water and drying). Dialuric acid monohydrate was isolated from the cooled filtrate in the usual manner.

Direct Preparation of Alloxantin (Only) from Alloxan at Room Temperature.—(*a*) *Below the "Critical" Concentration.*—Deaerated water (515 ml.) was saturated with hydrogen sulfide (1.7 Gm.) at room temperature. To this solution was added a freshly prepared solution of 16.01 Gm. of alloxan monohydrate in 785 ml. of deaerated water, and the flask rapidly stoppered. Sulfur was immediately liberated. Carbon disulfide (100 ml.) was added and the flask quickly stoppered. After standing overnight at room temperature, colorless crystals had separated, the odor of hydrogen sulfide had disappeared, and the aqueous layer was no longer opalescent. The mixture was filtered with slight suction (under nitrogen atmosphere) and the crystals washed with carbon disulfide and dried; wt. (dry), 12.2 Gm. (The carbon disulfide layer was separated, and evaporated to dryness; wt. of sulfur, 1.6 Gm.)

(*b*) *Above the "Critical" Concentration.*—Deaerated water (1,030 ml.) was saturated with hydrogen sulfide (3.4 Gm.), as above, and to the solution was added a freshly prepared solution of 32.02 Gm. of alloxan monohydrate in 270 ml. of deaerated water. After treatment as above, there was obtained 28.2 Gm. (dry) of colorless crystals (and 3.2 Gm. of sulfur).

Action of Excess Hydrogen Sulfide on Alloxantin Dihydrate.—(*a*) *At 25°.*—Dry, finely powdered alloxantin dihydrate (3.22 Gm.; 0.01 mole) was suspended in 1,110 ml. of deaerated water at 25°, and moist hydrogen sulfide was passed in until there was no further gain in weight. After stoppering the flask and keeping it overnight at room temperature, the undissolved material was filtered off and dried; wt., 0.32 Gm. (unchanged on extracting with boiling water and drying).

The experiment was repeated, using a magnetically stirred suspension of 16.1 Gm. of alloxantin dihydrate in 1,300 ml. of deaerated water. After saturation with hydrogen sulfide, the mixture was kept at room temperature for three days, with occasional stirring, and then filtered; wt. of sulfur, 1.60 Gm. The aqueous filtrate was cooled in ice, while passing in hydrogen sulfide, stoppered, and kept overnight in the refrigerator. The colorless needle-like crystals were filtered off under hydrogen sulfide, and dried under high vacuum; wt. (dry), 9.0 Gm. Examination of its infrared absorption spectrum (34) showed this material to be dialuric acid monohydrate.

The following method is more convenient. A suspension of 16.1 Gm. of alloxantin dihydrate in 1,300 ml. of water was prepared in a separatory funnel. After saturating the suspension with hydrogen sulfide, the free sulfur was extracted with two 100-ml. portions of carbon disulfide, and dialuric acid monohydrate, crystallized from the aqueous layer by cooling as above, was filtered off, under slight hydrogen sulfide pressure, and dried under high vacuum.

(*b*) *At the Boiling Temperature.*—Distilled water (600 ml.) was deaerated by boiling under reflux, and allowed to cool somewhat while hydrogen sulfide was passed in through the condenser. Dry alloxantin dihydrate (32.22 Gm.) was quickly added and the mixture reheated to boiling while hydrogen sulfide was passed in. After passing the gas for sixty minutes, reaction was judged to be complete; the mixture was then filtered hot by means of a filtering apparatus preflushed with hydrogen sulfide. The precipitate (sulfur) was dried in the vacuum desiccator; wt., 2.01 Gm. (unchanged by extraction with 250 ml. of boiling water under reflux and drying). (The reaction had therefore not proceeded to completion, possibly because of partial decomposition of alloxantin in the boiling solution.) The initial mother liquor was cooled in ice, while passing in hydrogen sulfide, stoppered, and kept overnight

in the refrigerator. The colorless crystals of dialuric acid monohydrate were filtered off under hydrogen sulfide, and dried under high vacuum; wt., 21.50 Gm.

(*c*) *At 89°.*—Because some decomposition apparently occurred under the above conditions, the reaction was repeated with the following modifications. After the water (600 ml.) had been deaerated, it was cooled in ice to room temperature while hydrogen sulfide was passed in to saturation. Finely powdered alloxantin dihydrate (32.22 Gm.) was then added, and hydrogen sulfide was passed in while the suspension was very slowly heated under reflux until all the alloxantin had dissolved, giving a clear, colorless solution (temp. 89°) with sulfur floating on the surface. The mixture was cooled to about 46° and carbon disulfide (100 ml.) was added in portions; the sulfur all dissolved and colorless crystals were deposited in the upper, aqueous layer. The mixture was now cooled in ice, while continuing the gas stream, filtered under hydrogen sulfide, and the crystals of dialuric acid monohydrate washed with carbon disulfide and dried under high vacuum; wt., 27.2 Gm. (The carbon disulfide layer was separated from the aqueous mother liquor, which was then washed with four 25-ml. portions of carbon disulfide. The carbon disulfide solution and washings were combined and evaporated to dryness, yielding sulfur; wt., 3.2 Gm.) This is a satisfactory method for preparing dialuric acid monohydrate from alloxantin dihydrate.

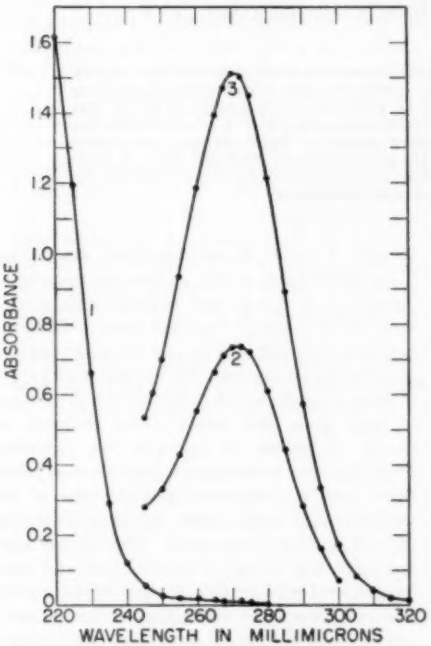

Fig. 1.—Ultraviolet absorption spectra.

1, 0.0002 *M* alloxan monohydrate in 0.03 *N* hydrochloric acid. *2*, solution *1* treated with excess hydrogen sulfide at 25° (less the absorbances of hydrogen sulfide and colloidal sulfur). *3*, 0.0002 *M* alloxantin dihydrate in 0.03 *N* hydrochloric acid treated as for solution *2*.

Ultraviolet Absorption Spectra of Alloxan and Dialuric Acid in Aqueous Solution.—In 1905, Hartley (36) discovered that the ultraviolet absorption spectrum of a freshly prepared aqueous solution of dialuric acid exhibits a peak at 270 mμ, whereas this peak is absent from the spectrum of aqueous alloxan solution. Freshly prepared aqueous alloxantin solution also showed a peak at 270 mμ. [These peaks disappeared if the solutions were kept; such stale solutions were studied by other workers (26, 37), who reported no peak.]

For confirmation of the results obtained on treatment of dilute aqueous solutions of alloxan and alloxantin with excess hydrogen sulfide, the ultraviolet absorption spectra were therefore studied. These spectra were obtained with a Cary recording spectrophotometer; the maximum error in the wave-length calibration of the instrument was ±5 Å.

As shown in Fig. 1, the height of the peak at 270 mμ observed on treating 0.0002 *M* alloxan monohydrate (in 0.03 *N* hydrochloric acid) with excess hydrogen sulfide was half that for 0.0002 *M* alloxantin dihydrate (in 0.03 *N* hydrochloric acid) similarly treated. This agrees with Eq. 3 and 4, respectively; it contradicts the work of Labes and Freisburger (38). Calculated as dialuric acid monohydrate, $\epsilon = 3{,}725 \pm 50$. [Patterson, *et al.* (29) found $\epsilon = 2{,}900$ in 10% sulfuric acid and $\epsilon = 3{,}200$ in 1% hydrochloric acid containing cysteine.] The curve for the untreated alloxan solution (Fig. 1) agrees with that in the literature (37).

SUMMARY

1. Treatment of aqueous alloxan solutions with excess hydrogen sulfide at room temperature yields dialuric acid (monohydrate), not alloxantin (dihydrate).

2. Because alloxan gradually decomposes in aqueous solution at the boiling temperature, the reduction of alloxan to dialuric acid is more satisfactorily performed at room temperature.

3. Alloxantin dihydrate is readily prepared by addition of 2 molar proportions of alloxan to 1 molar proportion of hydrogen sulfide (in aqueous solution).

4. Treatment of aqueous solutions or suspensions of alloxantin dihydrate with excess hydrogen sulfide yields dialuric acid (monohydrate). Some decomposition occurs at the boiling temperature, but at 89° or 25° the yield is excellent.

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The Determination of Analgetic Potency by a Quantal Method*,†

By BERNARD V. CHRISTENSEN and ARTHUR TYE

A modification of the Hardy-Wolff-Goodell radiant-heat method into a quantal procedure is described. Patterned after ED_{50} bioassays, this modification determines the fifty per cent analgesic dose (AD_{50}) of an analgetic when groups of white rats are exposed forty minutes after treatment with the analgetic to a selected stimulus (320 millicalories/sec./cm.^{2}) for the standard period of three seconds. Analgesic potency is measured by comparing the AD_{50} of an analgetic with the AD_{50} of morphine. The method is rapid and convenient and appears to be well suited for the screening and evaluation of analgetics.

THE Hardy-Wolff-Goodell (1) radiant-heat method has won wide acceptance as a reliable procedure for analgesimetric studies. However, it has generally been used to obtain data based on graded responses. Involving, as it does, the tedious step-wise determination of pain thresholds with varying successive intensities of stimulus both before and after treatment of the test animal with the analgetic, the procedure for obtaining this type of response is slow and time-consuming. Moreover, the data so obtained are difficult to evaluate because the exact relationship between rise in pain threshold and analgetic potency is subject to various interpretations.

The modification of the Hardy-Wolff method from a graded response procedure to a quantal one patterned after an ED_{50} quantal assay seems to offer a means of avoiding these drawbacks. This modification would involve the treating of successive groups of test animals with graded doses of an analgetic, exposing them, after a certain time interval, to a certain selected intensity of radiant-heat stimulus for the standard period of three seconds, and determining the percentage of each group that has attained analgesia as shown by failure to react to the stimulus. From the data so obtained a dose-per cent effect curve can be constructed and the dose of the analgetic that would cause analgesia in 50 per cent of animals determined. The 50 per cent analgesic dose (AD_{50}) of the analgetic can then be compared with the AD_{50} of a standard analgetic like morphine, and estimation of its potency can be made in terms of morphine. One advantage of such a procedure is that it effects a considerable saving of time and labor by not determining the pretreatment thresholds, as these are known to be normally distributed. Secondly, the actual determination of the after-treatment

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response is simple and clear-cut, requiring only a single observation, in contrast to the tedious step-wise determination of a pain threshold. Finally, the method of evaluation of results, the comparison of the ED_{50} of the best preparation with that of a reference standard, is one that is widely used in pharmacological assay work.

It is true that a quantal response gives less information per animal than a graded response and that, therefore, more animals are required to give an assay of equal reliability. But if, as in this case, the graded response determination involves complex measurement and computation, the drawback of requiring a greater number of animals is more than compensated for by the simplification of experimental procedure and interpretation of results.

In designing such a quantal procedure, the first essential is the selection of a suitable intensity of stimulus. For measuring analgesic potency, it would seem reasonable to choose one that would cause a degree of pain such as is commonly encountered clinically and of the order that analgetics are generally required to combat. Hardy, *et al.* (2), found that with radiant heat stimuli the pain threshold is reached at about 220 millicalories/sec./cm.^{2} and that stimuli greater than 680 millicalories/sec./cm.^{2} evolve ceiling pain. A stimulus of 320 millicalories/sec./cm.^{2} causes medium to severe pain and should therefore be a suitable stimulus. Hardy, *et al.*, found that the quality of pain sensations changes at about this point from a definite pricking sensation to one with an added burning quality. Another advantage of a stimulus of 320 millicalories/sec./cm.^{2} is that it is in the range of maximum discriminatory sensitivity, which extends approximately from 220 to 320 millicalories/sec./cm.^{2}

The second essential in designing a quantal assay is the selection of the optimum time interval between treatment and observation of response. In analgesimetry the response is probably best measured at the time of maximum effect. Time-action curves of various analgetics show that the peak effect generally occurs twenty minutes to one hour after injection. Experiment with various intervals during this period showed that forty minutes was the most efficient time interval.

The final procedure adopted was the application by a modified Hardy-Wolff-Goodell pain threshold apparatus of a stimulus of 320 millicalories/sec./cm.^{2} to the tails of white rats forty minutes after injection of an analgetic. Dose-per cent effect curves were obtained for various analgetics using five dose-groups of 12 rats each. The curves when plotted on log-probit paper were found to be satisfactory when tested for linearity and parallelism and gave satisfactory estimates of the potency of various analgetics and morphine solutions treated as unknowns. Using the same number of animals a three dose-group cross-over design appeared to be slightly more efficient and gave the following potency ratios with 19/20 confidence limits:

Drug	Potency Ratio
Morphine sulfate	1.0
Codeine phosphate	0.14 (0.13 to 0.15)
Methadone HCl	2.2 (2.0 to 2.4)
Metopon HCl	3.9 (3.6 to 4.3)
Meperidine HCl	0.24 (0.22 to 0.27)

The ED_{50} quantal method here described is a rapid and convenient procedure which appears suitable for the routine screening and evaluation of analgetics. It might also be used in the investigation of various problems associated with analgesia such as potentiation and addiction.

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Viscosity Studies on Calamine Lotion U. S. P. XIV*

By JOHN R. HOHMANN,† VISHNU N. BHATIA,† and LOUIS C. ZOPF‡

Certain inherent difficulties have been encountered in the large-scale manufacture of calamine lotion, U. S. P. XIV and in the preparation of phenolated calamine lotion N. F. IX. This report indicates that variations in the rate of settling of calamine and zinc oxide are due to variations in polyethylene glycol 400 monostearate from different sources and from different batches. A viscosity standard for the ester to correct this difficulty is suggested. The incorporation of excessive air in the course of preparation and the resulting shrinkage after packaging may be avoided by complete submersion of the stirrer and stirring at a slow speed. Increasing the polyethylene glycol 400 monostearate from 2 per cent to 4 per cent when phenol is to be added prevents loss of suspending power of the gel and the resulting separation that has frequently been experienced.

IN THE latest edition of the U. S. P. a new type of calamine lotion has been introduced which utilizes a base composed of polyethylene glycol 400 monostearate, polyethylene glycol, and water. This base serves as a suspending agent by virtue of its high viscosity. With the introduction of this improved formula for calamine lotion certain problems have arisen: (*a*) It has been noted that the rate of settling of the solid particles varies considerably in samples of the lotion prepared from different batches of polyethylene glycol 400 monostearate. (*b*) When phenolated calamine lotion N. F. is prepared, there is a great loss of suspending ability upon the addition of phenol.

The Stokes law which gives the relationship between viscosity and the rate of settling states that:

$$U = \frac{2}{9} \times \frac{r^2(\delta - \delta')g}{n}$$

where

U = rate of settling
r = radius of the particles
δ = density of the particles
δ' = density of the suspending agent
g = acceleration due to gravity
n = viscosity

Since the variation in the rate of settling of different samples persists even when the values of r, δ, δ', and g are kept constant, it can be reasonably assumed in the case of calamine lotion the rate of settling varies inversely as the viscosity. This may be an oversimplification of facts, since there are other factors (such as the degree of micelle formation by the ester) which may also contribute to the formation of good suspension.

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EXPERIMENTAL

This assumption was tested by determining the rate of settling of the calamine and zinc oxide in the lotions, and the viscosities of the corresponding gels prepared from different samples of the ester.

Determination of Rate of Settling.—Samples of the lotion were prepared using different esters and classified as follows:

****	good suspension
***	fair suspension
**	poor suspension
*	rapid settling

Preparation of the Gel and Determination of Its Viscosity.—Two grams of polyethylene glycol 400 monostearate was melted on a water bath and heated to a temperature of 70°. Boiling distilled water (90 cc.) was added to this and the mixture stirred with a mechanical stirrer, care being taken to stir at a low speed and to keep the blades of the stirrer submerged to prevent the inclusion of air. Polyethylene glycol 400 (8 cc.) was then added and the mixture gradually cooled to 25–28°, the stirring being continued during these processes. The gel thus obtained is poured into a Hoeppler Viscometer and its viscosity determined at 20°. The gel should not be cooled below 25°, otherwise it is difficult to pour it into the viscometer without the inclusion of air bubbles. However, if it is poured before it has cooled to 28°, the gel formation is hindered and the viscosity values obtained are lower.

The following results were obtained:

Sample No. (Stearate)	Rate of Settling of Calamine Lotion	Viscosity of Corresponding Gel
1	**	73.0 c. p. s.
2	****	132.6 c. p. s.
3	***	96.0 c. p. s.
4	****	101.7 c. p. s.
5	****	113.0 c. p. s.
6	*	22.0 c. p. s.
7	*	35.3 c. p. s.

The first 5 samples meet the existing U. S. P. standards.

Sample 3 was used in the preparation of the phenolated lotion. On the addition of 1% phenol the lotion settled rapidly and the viscosity of the gel dropped from 96 c. p. s. to 5 c. p. s.

The viscosity values for the gels prepared from ester sample 3 using different amounts of the ester are:

2% ester with 1% phenol	5 c. p. s.
2.5% ester with 1% phenol	7 c. p. s.
3% ester with 1% phenol	69 c. p. s.
3.5% ester with 1% phenol	128 c. p. s.

DISCUSSION

It is evident from the above data that there is a wide variation in the viscosities of the gels prepared from different samples of the ester. It is also evident that there is a relatively small difference in the viscosities of the gels prepared from the esters which meet the U. S. P. requirements. It appears that if the esters are reasonably pure, it should be possible to reproduce the desired viscosity within reasonable limits. Despite this, it was thought advisable to determine a viscosity minimum for the gel and thus establish a viscosity standard for the ester.

The rates of settling of calamine and zinc oxide in lotions of different viscosities were used as the basis for determining the viscosity minimum. The results indicate that the viscosity minimum is in the vicinity of 75 c. p. s.; that is, a 2% gel prepared from an ester should not have viscosity lower than 75 c. p. s.

At present the value of such a viscosity standard is limited, due to the following reasons:

(*a*) The rate of settling is also dependent on particle size, and the U. S. P. does not specify any requirements for the particle size of either calamine or zinc oxide.

(*b*) The amount of calamine lotion prepared also appears to affect the rate of settling to some degree; the larger lots settling at a faster rate than the smaller ones. This is probably due to the larger conglomerates present in the large lots, as it is more difficult to obtain a uniform dispersion of the solid particles when working with large quantities.

(*c*) Another factor which may affect the suspension is the presence of any incorporated air in the lotion. This can be avoided by having the stirrer completely submerged during the preparation of the lotion and by stirring at a relatively low speed.

In connection with the difficulty encountered in the preparation of the phenolated calamine lotion N. F., it is obvious from the data represented that the loss of suspending power is due to decrease in viscosity caused by the addition of phenol. It has been established that the ester is soluble in phenol. Therefore, upon addition of phenol, it tends to form a simple solution rather than a gel. This difficulty can be overcome by adding additional amounts of ester which will exceed the solubility of the ester in the phenol solution and re-establish conditions for the formation of a gel. In view of the above, for the preparation of the phenolated lotion, the ester should be increased from 2% to 3.5 or 4%. Thus, calamine lotion U. S. P. which contains only 2% of the ester does not form a suitable base for the addition of phenol and instead a lotion with the increased amount of ester should be used.

The Preparation of Some Dialkylcyanoacetamides*,†

By ROBERT F. DOERGE‡ and CHARLES O. WILSON§

A general procedure for the synthesis of dialkylcyanoacetamides and methods for the preparation and characterization of eight cyanoacetamides are described.

IN CONNECTION with certain studies being carried on, it was necessary to prepare various dialkylcyanoacetamides. Of the various methods (1–11), that of Errera (1) seemed the most desirable, since good yields of a fairly pure product were given on the few dialkylcyanoacetamides prepared by this method.

The authors were interested in finding a procedure which would be of general application in the preparation of various dialkylcyanoacetamides, and have applied the aforementioned method (1) with slight modifications to the preparation of known monoalkylcyanoacetamides and also to the new dialkylcyanoacetamides reported herein.

The method used by Errera (1) was of value only where the two alkyl groups are the same. He also reported a 77 per cent yield of diethylcyanoacetamide; in the present work it was found to be impossible to attain this yield using his procedure or the present modifications.

The most important modification of the conventional alkylation procedure was that introduced by Phillips (12). This modification consists of the addition of a solution of sodium ethoxide in anhydrous alcohol to a mixture of

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† Abstracted from a thesis presented to the Graduate Faculty of the University of Minnesota by Robert F. Doerge in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

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the cyanoacetamide to be alkylated and the alkyl halide in anhydrous alcohol at such a rate that a drop of the reaction mixture, applied to phenolphthalein paper, never causes more than a very slight pink coloration.

By avoiding prolonged contact between the original cyanoacetamide and the sodium ethoxide, it was possible to minimize hydrolysis, and the yield of the alkylated cyanoacetamide was increased. This modification also made the purification easier. However, in using either this modified method or the method reported by Errera (1), it was never possible to attain his reported yield for diethylcyanoacetamide. Christensen (15) reported the monoalkylcyanoacetamides but not the yields so it was impossible to make a comparison with those obtained in the present paper. Hessler (16) reported the synthesis of isoamylcyanoacetamide, but here again the yield was not reported.

Pertinent data for the compounds synthesized are given in Table I.

EXPERIMENTAL[1]

Apparatus.—The reaction apparatus used in this work consisted of a one-liter, three-necked flask equipped with mechanical stirrer inserted through a mercury seal, a dropping funnel fitted with a capillary tip to allow the formation of small drops, and a reflux condenser fitted with a calcium chloride tube to protect the system from atmospheric moisture.

Method.—The cyanoacetamide or monoalkylcyanoacetamide was placed in the reaction flask with anhydrous alcohol (250 cc.) (13) and dissolved by heating on a water bath. The alkyl bromide was then added all at once through the dropping funnel. While keeping this solution at the refluxing temperature and well stirred, a solution of sodium ethoxide, which had been previously prepared by dissolving the sodium in anhydrous alcohol (250 cc.) was added dropwise. The sodium ethoxide solution was added at the rate of about two drops per second. This addition required about two and one-half hours. The stirring and heating were continued for another one and one-half hours, making a total of four hours for the reaction. The reaction mixture was then allowed to stand overnight at room temperature. The precipitated sodium bromide was then filtered off. The filtrate was then decolorized by Norit, if necessary, and concentrated under reduced pressure by using the water pump and a water bath. The concentrate was then diluted with water until it became cloudy, and was placed in the refrigerator overnight to allow crystallization. The product was recrystallized from either hot water or from a mixture of alcohol and water.

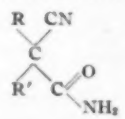
Cyanoacetamide.—Cyanoacetamide was obtained from commercially available ethyl cyanoacetate (Eastman Kodak Co.) by the method in "Organic Syntheses" (14).

Ethylcyanoacetamide.—This amide was prepared according to the general method from cyanoacetamide (42 Gm.), ethyl bromide (65.4 Gm.), and sodium (11.5 Gm.). The yield was 53.5% and the product, recrystallized from hot water, melted at 112–113°. It had been reported in the literature as 113° (15).

Isopropylcyanoacetamide.—This compound was synthesized from cyanoacetamide (42 Gm.), isopropyl bromide (73.8 Gm.), and sodium (11.5 Gm.). A yield of 51% was obtained and the product was recrystallized from hot water. The melting point was 124–125° and was reported in the literature as 125° (15).

[1] All melting and boiling points are uncorrected.

TABLE I.—DERIVATIVES OF CYANOACETAMIDE



R	R′	M. P., °C.	Yield, %	Molecular Formula	Analyses, % Calcd.	Found
C_2H_5	H	112–113[a]	53.5	$C_5H_8N_2O$		
i-C_3H_7	H	124–125[a]	51	$C_6H_{10}N_2O$		
t-C_4H_9	H	90–92[a]	40	$C_7H_{12}N_2O$		
i-C_5H_{11}	H	139–140[b]	72.7	$C_8N_{14}N_2O$		
C_2H_5	C_2H_5	120–121[c]	63.1	$C_7H_{12}N_2O$		
C_2H_5	*i*-C_3H_7	111–111.5	67.5	$C_8H_{14}N_2O$	C, 62.30	62.28
					H, 9.15	8.90
					N, 18.17	18.23
C_2H_5	*i*-C_4H_9	127.5–128.5	68.7	$C_9H_{16}N_2O$	C, 64.25	67.36
					H, 9.59	10.22
					N, 16.65	14.81
C_2H_5	*i*-C_5H_{11}	68–69	58	$C_{10}H_{18}N_2O$	C, 65.89	65.85
					H, 9.96	9.70
					N, 15.38	15.19

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[b] Hessler, J. C., *J. Am. Chem. Soc.*, **35**, 993(1913).
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Isobutylcyanoacetamide.—This compound was prepared from cyanoacetamide (42 Gm.), isobutyl bromide (82.2 Gm.), and sodium (11.5 Gm.). The yield was 40% and the crude material, recrystallized from dilute alcohol (25%), melted at 90–92°. Previous workers reported a melting point of 93° (15).

Isoamylcyanoacetamide.—This amide was prepared from cyanoacetamide (42 Gm.), isoamyl bromide (90.6 Gm.), and sodium (11.5 Gm.). The yield was 72.7% and the product, recrystallized from dilute alcohol (25%), melted at 139–140°, as compared with 142° reported in the literature (16).

Diethylcyanocetamide.—This amide was prepared from ethylcyanoacetamide (56 Gm.), ethyl bromide (65.4 Gm.), and sodium (11.5 Gm.). The yield was 63.1% and the crude product, when recrystallized from hot water, melted at 120–121°. The literature reports 121° (1).

Ethylisopropylcyanoacetamide.—This compound was prepared from isopropylcyanoacetamide (63 Gm.), ethyl bromide (65.4 Gm.), and sodium (11.5 Gm.). A yield of 67.5% was obtained, and when recrystallized from alcohol (25%) it had a melting point of 111 to 111.5°.

Anal.—Calcd. for $C_8H_{14}N_2O$: C, 62.30; H, 9.15; N, 18.17. Found: C, 62.28; H, 8.90; N 18.23.

Ethylisobutylcyanoacetamide.—This amide was prepared using isobutylcyanoacetamide (70.1 Gm.), ethylbromide (65.4 Gm.), and sodium (11.5 Gm.). The yield was 68.7% and after recrystallization from alcohol (25%) it melted at 127.5 to 128.5°.

The analysis permits some uncertainty as to the identity of the compound. However, this compound, when used in further synthesis, gave a compound which yielded a derivative of the known and desired structure. (To be submitted for later publication.)

Anal.—Calcd. for $C_9H_{16}N_2O$: C, 64.25; H, 9.59; N, 16.65. Found: C, 67.36; H, 10.22; N, 14.81.

Ethylisoamylcyanoacetamide.—This compound was synthesized from isoamylcyanoacetamide (77.1 Gm.), ethyl bromide (65.4 Gm.), and sodium (11.5 Gm.). The yield was 58% and after recrystallization from alcohol (50%) it melted at 88–89°.

Anal.—Calcd. for $C_{10}H_{18}N_2O$: C, 65.89; H, 9.96; N, 15.38. Found: C, 65.85; H, 9.70; N, 15.19.

SUMMARY

1. A procedure for the synthesis of dialkylcyanoacetamides has been presented.

2. Eight substituted cyanoacetamides were prepared:

(*a*) Ethylcyanoacetamide.
(*b*) Isopropylcyanoacetamide
(*c*) Isobutylcyanoacetamide
(*d*) Isoamylcyanoacetamide
(*e*) Diethylcyanoacetamide
(*f*) Ethylisopropylcyanoacetamide
(*g*) Ethylisobutylcyanoacetamide
(*h*) Ethylisoamylcyanoacetamide

Those listed as "*f*," "*g*," and "*h*" have not been previously reported.

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Composition of Gum Turpentines of Pines: A Report on *Pinus echinata, P. rigida,* and *P. ponderosa* from Utah*

By N. T. MIROV

THE PRESENT paper is a continuation of an investigation (1) of the oleoresin present in species of the genus *Pinus*, and deals with composition of the following pines: *Pinus echinata, P. rigida*, and *P. ponderosa*.

PINUS ECHINATA

Pinus echinata Mill. is commonly known as shortleaf pine. It grows from New York to Florida and west through West Virginia, southwestern Illinois, and eastern Tennessee to sourthern Missouri, Arkansas, Oklahoma, and eastern Texas. This pine has been considered a poor source of commercial oleoresin products, and consequently its turpentine has not been thoroughly investigated. There is in the Forest Service files, however, a typewritten report by Herty (2) on his analysis of the composition of shortleaf pine turpentine.

The oleoresin used by Herty was collected in northern Florida. Herty obtained the following data for two samples of crude shortleaf pine turpentine:

Percentage of volatile oil..	16.27 and 20.00%
Density (no temp. given).	0.8452 and 0.8464
Index of refraction (no temp. given).....	1.4728 and 1.4723
Optical rotation.....	+103° 49′ and +103° 49′

A rather low specific gravity of the turpentine caused Herty to suggest that it consists of a mixture of limonene and pinene, the former being present in a considerable excess. Fractional distillation of 100 cc. of the two samples combined gave the following results:

77% boiled at 172–175°
10% boiled at 175–177°
10% boiled at 177–187°
3% was residue above 187°

The oleoresin used in the present experiment was obtained through the courtesy of Keith W. Dorman, of the U. S. Forest Service. It was collected in Bent Creek Experimental Forest, near Asheville, N. C. Turpentine was obtained from the oleoresin under reduced pressure, so that at the end of distillation the temperature inside the flask was 150°, and pressure was 0.1 mm. The yield of turpentine amounted to 21.4%.[1] The physical characteristics of the turpentine were as follows:

Density d_4^{20}.....	0.8575
Index of refraction n_D^{23}.....	1.4685
Optical rotation in 10-cm. tube $[a]_D^{23}$...	+8°30′
Optical rotation in 5-cm. tube $[a]_D^{23}$....	+4°15′

It is seen that the physical characteristics of shortleaf pine turpentine differ considerably from those obtained by Herty.

A trial distillation of 20 Gm. of the turpentine under atmospheric pressure showed that 60% distilled at 155°, and 40% at 160–163°. Very little residue boiling above 163° was left in the flask. A batch of 333 Gm. of turpentine was then subjected to fractional distillation at 85 mm. pressure, using a heated-jacket distilling column of a Vigroux type, 80 cm. long, equipped with a partial reflux condenser. A reflux ratio of approximately 1:10 was maintained. The results of this fractional distillation are shown in Table I.

α-Pinene was identified in fraction 1 by preparing a nitrosochloride in the following manner: 6 cc. of the turpentine, 10 cc. of amyl nitrite, and 15 cc. of glacial acetic acid were mixed; the mixture was cooled in an ice-salt bath, and 11 cc. of 1:1 mixture of hydrochloric and glacial acetic acids was added, drop by drop. A copious precipitate of the α-pinene nitrosochloride was recrystallized twice from chloroform by addition of cold methanol. Melting point of the nitrosochloride was 103°. When mixed with known α-pinene nitrosochloride, it showed no depression of the melting point. Nitrosopiperidine was prepared from the nitrosochloride by dissolving and heating 5 Gm. of the latter in 4 cc. of piperidine and 4 cc. of 100% ethanol. After cooling, the solution was diluted with three volumes of cold water and cooled overnight in a refrigerator. The solidified oil was recrystallized twice from 95% ethanol. The nitrosopiperidine melted at 118–119°.

β-Pinene was identified in fraction 7 by preparing nopinic acid as follows: 7 Gm. of the oil was shaken for one-half hour in 600 cc. of water to which were added 24 Gm. of potassium permanganate and 5 Gm. of sodium hydroxide. The unreacted oil was removed by steam distillation, and the liquid was filtered and evaporated under reduced pressure to 200 cc. Sodium nopinate precipitated on cooling. The sodium nopinate was dissolved in hot water, and nopinic acid was released by adding normal hydrochloric acid and extracting with ethyl ether. After recrystallization from benzene, the nopinic acid had a melting point of 126–127°.

No limonene was detected in the redistilled residue of the fractional distillation (Table I).

The distillation behavior shown in Table I, the physical constants of the fractions, and the chemical identification of the components show that the shortleaf pine turpentine consisted of about 85% *d*-α-pinene, 11% *l*-β-pinene, and 4% residue, com-

* Received January 29, 1951, from the California Forest and Range Experiment Station, which is maintained by the Forest Service, U. S. Department of Agriculture, in cooperation with the University of California, Berkeley.

[1] Emery F. Kimbrough assisted in distilling turpentine of shortleaf pine, as well as of several other pines.

TABLE I.—FRACTIONAL DISTILLATION OF 333 GM. OF PINUS ECHINATA TURPENTINE AT 85 MM. PRESSURE

Fraction	Temperature, °C.	Distillate		Optical Rotation, $[\alpha]_D^{23}$	Index of Refraction n_D^{23}
		Gm.	%		
1	86–87	270.8	81.2	+13°20′	1.4658
2	87–88	4.3	1.3	+4°00′	1.4672
3	88–89	8.1	2.4	+1°20′	1.4670
4	89–90	5.2	1.6	−1°00′	1.4710
5	90–91	6.4	1.9	−6°00′	1.4722
6	91–92	7.7	2.3	−9°40′	1.4732
7	92–94	8.5	2.6	−12°50′	1.4742
8	94–95	8.1	2.5	−16°20′	1.4752
Residue	Over 95	13.9	4.2		
Total		333.0			

posed probably of polymerized terpenes and a small quantity of high-boiling fractions. It is difficult to explain why the turpentine samples analyzed by Herty and by the present writer should differ so much in their composition. In view of past experience (3), it seems highly improbable to expect in the same species a predominance of limonene in one sample and a total absence in another, unless the sample is obtained from a natural hybrid between two chemically different forms. A further inquiry into this discrepancy is desirable.

PINUS RIGIDA

Pinus rigida Mill. is commonly known as pitch pine. Its range extends from New Brunswick and southern Ontario, south to Georgia and Tennessee. Closely related to this pine, morphologically, is *P. rigida* var. *serotina* Michx., or pond pine; this latter pine grows on the Coastal Plain from New Jersey to Florida and Alabama. Some botanists consider pond pine a separate entity, *P. serotina*. It is distinguished from pitch pine by its more resinous winter buds, much longer leaves (6–8 and even 10 in. as compared with $3^1/_2$–$4^1/_2$ in. of pitch pine leaves) and less prickly, more rounded cones, which are serotinous, that is, they remain closed for many years. Composition of the pond pine turpentine was studied in 1908 by Herty and Dickson (4) and reported to consist almost entirely (80–90%) of *l*-limonene.

The oleoresin sample from pitch pine was collected for the present analysis in 1949 by Keith W. Dorman near Asheville, N. C. The turpentine was obtained by heating the oleoresin under reduced pressure, so that at the end of distillation the temperature of the oleoresin was 200°, and pressure was 0.1 mm. Turpentine obtained in this way amounted to 23% of the weight of the oleoresin and possessed the following physical characteristics:

Density d_4^{20}	0.8560
Index of refraction n_D^{23}	1.4701
Optical rotation $[\alpha]_D^{23}$	−7°40′
Optical rotation in 5-cm. tube	−3°50′

A batch of 380 Gm. of turpentine was fractionated in the previously described 80-cm. column at reduced pressure into three large fractions. Fraction A, amounting to 207 Gm., boiled between 41 and 45° at 20 mm. pressure. Fraction B, amounting to 131 Gm., boiled at 45–59° at 20 mm. pressure; and fraction C, amounting to 42 Gm., boiled at 59–72° at 35 mm. pressure. Fraction A was redistilled under atmospheric pressure with results shown in Table II. α-Pinene was identified in all three fractions (of the large fraction A) by preparation of nitrosochloride (melting point 105°) and of nitrosopiperidine (m. p. 118–119°), as outlined earlier in this article.

The second large fraction (B), having a boiling range between 45 and 59° at 20 mm. pressure and optical rotation range between −11° and −18°, was also redistilled under atmospheric pressure. Fifty per cent of the fraction distilled below 157° and is regarded as consisting of α-pinene; 20% distilled at 157–160°; and 25% distilled between 160 and 167°. Flask residue boiling above 167° amounted to 5%. β-Pinene was identified in the fractions boiling between 160 and 167° by preparation of nopinic acid of melting point 126°–127°.

The third fraction (C), also levorotatory, boiling at between 59 and 72° at 35 mm. pressure, and the flask residues of distilling fractions A and B—a total of 46 Gm.—were fractionated under atmospheric pressure. Results obtained are shown in Table III. Fraction 4 (b. p. 174–175°) was brominated and crystalline tetrabromide was obtained, which, after repeated recrystallization from ethyl ether, melted at 104–105°. It is concluded that fraction C consisted of *l*-limonene. The residue was not large enough to suspect any appreciable quantity of sesquiterpenes. To sum up, turpentine of *Pinus rigida* consists of about 65% *l*-α-pinene; 20% *l*-β-pinene; and 10% *l*-limonene. Sesquiterpenes are apparently not found.

TABLE II.—RESULTS OF REDISTILLATION OF FRACTION A UNDER ATMOSPHERIC PRESSURE

Fractions	B. P., °C.	Oil, Gm.	n_D^{23}	d_4^{23}	$[\alpha]_D^{23}$
1	154–155	165.0	1.4660	0.8575	−2°0′
2	155–156	14.1	1.4696	0.8583	−5°20′
3	156–157	3.5	1.4714		−8°10′
Residue	Above 157	8.9			

TABLE III.—RESULTS OF FRACTIONATION, UNDER ATMOSPHERIC PRESSURE, OF FRACTION C AND FLASK RESIDUES OF FRACTIONS A AND B

Fractions	B. P., °C.	Oil, Gm.	Optical Rotation $[\alpha]_D^{25}$
1	171–172	12	..
2	172–173	13	−32°20′
3	173–174	4	..
4	174–175	10	−34°00′
5	175–176	2	..
6	176–180	2	..
Residue and losses		3	..

PINUS PONDEROSA FROM SOUTHWESTERN UTAH

Pinus ponderosa occupies a large area of North America, chiefly in the West. There are several varieties of this pine, some known, others suspected. The variety growing on the west slope of the Sierra Nevada has been recently investigated as to the composition of its turpentine (1). The turpentine of this variety is always levorotatory; it consists of 51% of *dl*- and *l*-β-pinene, 20% *dl*- and *l*-Δ³-carene, 25% *l*-limonene and dipentene; 3% *dl*- and *d*-cadinene, and 0.5% a new sesquiterpene, which is characterized by a trihydrochloride having melting point of 129–130°. No α-pinene was found.

Turpentine of the Rocky Mountain variety of *Pinus ponderosa* was analyzed by Schorger in 1911–1913 (5). The turpentine was dextrorotatory, consisting of: 60–70% *d*-α-pinene; 20% *l*-β-pinene; and 20–25% limonene.

The oleoresin used in the present experiment was obtained from 20 mature trees in two localities in the Wasatch Mountains, Utah. One locality was near Panguitch Lake, at an elevation of 8,500 feet; the other was at Dave's Hollow Ranger Station within the Dixie National Forest. Oleoresin samples of individual trees were subjected to distillation under reduced pressure so that at the end of the operation, temperature in the flask was 175° and pressure was 0.1 mm. In average, the yield of turpentine was 26.5%.

Individual trees varied in the optical properties of their turpentine; some were dextrorotatory, others were levorotatory. Optical activity varied from +23°20′ to −17°40′.[2] In this respect turpentine of the Utah variety of ponderosa pine resembled that of the pine growing on the western side of the Great Basin, near Mt. Rose, Nevada, and described as *Pinus washoensis* (1, 6).

A composite sample of turpentine from Utah had the following characteristics:

Density d_4^{25}	0.8611
Index of refraction n_D^{25}	1.4720
Optical rotation $[\alpha]_D^{25}$	+6.0°

A batch of 400 Gm. of turpentine was fractionated under reduced pressure in a flask equipped with the 80-cm. column. A reflux ratio of approximately 1:10 was maintained. Results of the fractional distillation are presented in Table IV.

Identification of *d*-α-Pinene.—Fractions 1 and 2 were redistilled under atmospheric pressure over sodium. Most of the oil distilled between 157 and 160°. α-Pinene was identified by the preparation of α-pinene nitrosochloride, as explained earlier in this article. A copious precipitate of nitrosochloride was obtained; upon purification it melted at 105–106°. The nitrosochloride was then converted into α-pinene nitrosopiperidine; recrystallized from 95% ethyl alcohol, it melted at 118–119°. Attempts to identify β-pinene in fractions 2, 3, and 4 by preparing nopinic acid have failed. These fractions consisted of a mixture of α-pinene and Δ³-pinene.

Identification of *d*-Δ³-Carene.—A sample (5 Gm.) of fraction 4 was mixed with 2 cc. of glacial acetic acid and 4 Gm. of amyl nitrite, cooled in ice, and treated with 3.5 Gm. of nitric acid. Nitrosate was formed in abundance. After two recrystallizations by methyl alcohol from chloroform, the nitrosate melted at 142–143°. It did not depress the melting point of an authentic sample of Δ³-carene

[2] The subject of variation of optical properties and chemical composition of turpentine of individual trees in a population has not been explored. Studies in this direction may prove very profitable, both from a scientific and a practical point of view.

TABLE IV.—FRACTIONAL DISTILLATION OF 400 GM. OF UTAH VARIETY OF PINUS PONDEROSA UNDER 20 MM. OF PRESSURE

Fraction	Temperature, °C.	Distillate, Gm.	Index of Refraction n_D^{25}	Density d_4^{25}	Optical Rotation $[\alpha]_D^{25}$
1	57–59	103.4	1.4677	0.8564	+18°30′
2	59–61	71.5	1.4710	0.8552	+9°00′
3	61–62	5.0	....	....	....
4	62–64	130.5	1.4721	0.8543	+3°40′
5	64–65	9.0	....	....	....
6	65–66	16.5	1.4725	0.8535	±0°0′
7	66–67	5.0	....	....	....
8	67–68	4.0	....	....	....
9	68–69	8.0	1.4742	0.8523	−9°50′
10	69–74	3.0	....	....	....
11	74–80	3.0	....	....	....
12	80–104	10.5	1.4845	0.9039	−2°10′
Residue		27.6	....	....	....
Losses		3.0	....	....	....
Total		400.00			

TABLE V.—COMPARISON OF THREE FRACTIONS OF PONDEROSA PINE TURPENTINE WITH THOSE OF LONGIFOLENE

Material	Boiling Range, °C.	Density, d	Index of Refraction n_D	Specific Rotation $[\alpha]_D$	M. P. of Hydrochloride, °C.
Longifolene from *Pinus longifolia*	150–151° at 36 mm.	0.9284^{25}_{25}	1.4950^{30}	+42.73°	59–60
Longifolene from *Pinus torreyana*	62–64° at 0.2 mm.	0.9270^{24}_{4}	1.4940^{27}	Not given	59–60
Fraction 12 of Utah *Pinus ponderosa*	80–104° at 20 mm.	0.9039^{23}_{4}	1.4845	−2.88°	59–60
Fraction A of redistilled residue of Utah *Pinus ponderosa*	140–145° at 30 mm.	0.9268^{23}_{4}	1.5016^{23}	−7.91°	59–60
Fraction B of redistilled residue of Utah *Pinus ponderosa*	130–140° at 15 mm.	0.9243^{23}_{4}	1.5027^{23}	−14.52°	59–60

nitrosate. Therefore, it was concluded that the precipitate obtained was Δ^3-carene nitrosate, indicating the presence of large amounts of Δ^3-carene in the turpentine.

Limonene was identified in fraction 9 with some difficulty. Four grams of the oil was dissolved in 8 Gm. of glacial acetic and treated drop by drop with bromine dissolved in four volumes of glacial acetic acid until a persistent yellow color appeared. Tetrabromide precipitated only after a prolonged standing of the treated oil in the refrigerator. Melting point of the tetrabromide, recrystallized from ethyl ether, was 104–105°.

Identification of Longifolene.—A sample (3 Gm.) of fraction 12 dissolved in 18 cc. of absolute ethyl ether was treated with dry hydrogen chloride and kept in a stoppered flask in a refrigerator for one week. Then the mixture was poured on a watch glass and the solvent was permitted to evaporate. The oily residue was put into the refrigerator again; in one week the oil solidified; the solid was then pressed out on porous clay plate and the hydrochloride crystals were recrystallized twice from glacial acetic acid.

The fraction 12 yielded a small amount of crystals which melted at 59–60°. Better results were obtained when flask residue (see Table IV) was redistilled under reduced pressure. Two fractions yielded copious crystals of hydrochloride; a fraction boiling at 140–145° at 30 mm. pressure and having density $d^{23}_4 = 0.9268$, index of refraction $n^{23}_D = 1.5016$, and optical rotation $[\alpha]^{23}_D -7°20'$; and a fraction boiling at 130–140° at 15 mm. pressure and having density = 0.9243^{23}, index of refraction = 1.5047^{23}, and optical rotation −14°00′. The crystals of hydrochloride melted at 59–60°. When two drops of the fraction boiling at 130–140° (15 mm. pressure) were dissolved in 2 cc. of acetic anhydride and treated with one drop of concentrated sulfuric acid the mixture turned faint green.

In Table V the physical constants of three fractions of ponderosa pine turpentine are compared with those of longifolene.

Judging from this comparison, the sesquiterpene present is apparently longifolene, discovered by Simonsen (7) in *Pinus longifolia* of India and since described also as a component of Torrey pine turpentine (8).

Cadinene apparently is not found in turpentine of the Utah variety of ponderosa pine.

To sum up, turpentine obtained from ponderosa pine of the Wasatch Mountains of southwestern Utah consists of about 45% *dl*- and *d*-α-pinene, 40% *dl*- and *d*-Δ^3-carene, 2–3% *l*-limonene, and 10% sesquiterpene, *l*-longifolene, the remaining 5% being polymerized carene. This turpentine differs a great deal from the turpentine obtained on the west slope of the Sierra Nevada, inasmuch as the Utah turpentine does not contain β-pinene. On the other hand α-pinene, which is totally absent in the California turpentine, comprises about one-half of the Utah ponderosa pine turpentine. By investigating the turpentines of ponderosa pine from different localities of its extensive range, it is hoped eventually to clarify the problem of variability in composition of essential oil within a species.

It is probable that in individual trees, proportion of α-pinene and carene varies considerably.

SUMMARY

Chemical composition of turpentine of *Pinus echinata* from North Carolina was found to be as follows:

d-α-pinene	85%
l-β-pinene	11%

Pinus rigida turpentine, also from North Carolina, consisted of:

l-α-pinene	65%
l-β-pinene	20%
l-limonene	10%

Turpentine of *Pinus ponderosa* from southwestern Utah had the following composition:

dl- and *d*-α-pinene	45%
dl- and *d*-Δ^3-carene	40%
l-limonene	2–3%
Sesquiterpene *l*-longifolene	10%

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Notes

A Note on the Hydrolysis of Salicylamide*

By DONALD C. BRODIE and IVAN J. SZEKELY†,‡

SALICYLAMIDE (salamid, *o*-hydroxybenzamide) was first prepared (1) more than 100 years ago although it has never been accepted as a medicinally useful salicylic acid derivative. This fact may be attributed largely to the lack of knowledge regarding the drug's toxicity and pharmacologic action, which have only in recent years been studied. Since a demonstrable analgetic potency and a safe therapeutic index are now recognized, considerable interest has been shown in the possible therapeutic usefulness of salicylamide.

In 1891, Nesbitt (2) suggested that salicylamide might be a suitable substitute for salicylic acid and substantiated his report by including observations that blood pressure and respiration in man were not affected by therapeutic doses of the drug. The absorption of salicylamide from the gastrointestinal tract was reported by Bass in 1890 (3), and Bondzynski in 1897 (4).

Hydrolysis of salicylamide by tissue extracts has been reported (5, 6), with Gonnermann (6) demonstrating cleavage of the acid-amide linkage by extracts of sheep kidney. The fact that glucuronic acid excretion in dogs is increased following the administration of salicylamide was reported by Kase and Seki (7). Ichniowski and Hueper (8), Hart (9), and Litter, *et al.* (10), have contributed more recent studies regarding the pharmacology, toxicity, and possible clinic usefulness of the drug. Such data suggest that salicylamide might be an effective and useful analgetic agent, which, because of the stability of the acid-amide linkage, would not cause the gastric irritation that is sometimes encountered with other salicylates. During the course of formulation studies with the drug, a number of *in vitro* hydrolysis experiments were conducted to determine the extent of hydrolysis under conditions simulating those that might be encountered *in vivo*.

TABLE I.—ACID AND ALKALINE HYDROLYSIS OF SALICYLAMIDE *in Vitro*

Sample (1 Gm.)	Undissolved Salicylamide	Extracted[a] Salicylamide	Extracted Salicylic Acid	% Recovered
Acid Hydrolysis (Artificial Gastric Juice, *p*H 1.1)				
1. 4-hour digestion	0.844	0.120	Trace	96.4
2. 4-hour digestion	0.863	0.121	Trace	98.4
3. 4-hour digestion	0.808	0.143	Trace	95.1
			(mean)	96.6
			S[b]	1.7
Alkaline Hydrolysis (Artificial Pancreatic Juice, *p*H 8.2)				
1. 4-hour digestion	0.763	0.173	Trace	93.6
2. 4-hour digestion	0.810	0.135	Trace	94.5
3. 4-hour digestion	0.871	0.078	Trace	94.9
			(mean)	94.3
			S[b]	0.7
1. 8-hour digestion	0.818	0.127	Trace	94.5
2. 8-hour digestion	0.806	0.114	Trace	92.0
			(mean)	93.3
			S[b]	1.8
Control for Extraction of Known Amount Dissolved Salicylamide				
1. Sample (0.150 Gm.)		0.140		93.3
2. Sample (0.150 Gm.)		0.142		94.7
3. Sample (0.150 Gm.)		0.144		96.0
			(mean)	94.7
			S[b]	1.3

[a] Identified by melting point.
[b] Standard deviation.

Salicylamide is a white or slightly pink crystalline substance melting at 140° (11); it is slightly soluble in cold water, and soluble in hot water, alcohol, chloroform, and ether (12). An aqueous saturated solution has a *p*H of about 5.5.

* Received April 17, 1951, from the University of California College of Pharmacy, The Medical Center, San Francisco 22, Calif.
† Present address: Barnes-Hind Laboratories, Inc., San Francisco, Calif.
‡ The authors wish to acknowledge the assistance of Mr. S. J. Dean of the Manufacturing Laboratory of the University of California College of Pharmacy.

EXPERIMENTAL

Acid Hydrolysis.—One-gram samples of finely powdered salicylamide (m. p. 138–139°, uncorrected) were added to 40-cc. portions of artificial (13) gastric juice (*p*H 1.1) in round-bottomed centrifuge tubes, and suspended horizontally from a mechanical shaker in a constant temperature bath at 37.5°. The samples were shaken at a moderate rate during a four-hour digestion period. After cooling the tubes in ice, 4.1 Gm. of $NaHCO_3$ was added to each, thereby adjusting the *p*H to approximately 8. Undissolved salicylamide was removed by suction and washed with sufficient cold water, air-dried, and weighed. The volume of filtrate was adjusted to 90 cc. Removal of the dissolved salicylamide was accomplished by extraction of the alkaline filtrate with ether. Following acidification of the filtrate with 0.75 cc. of concentrated hydrochloric acid, the liberated salicylic acid was extracted with 5–35 cc. portions of ether, dried over Drierite, and evaporated to dryness.

Alkaline Hydrolysis.—One-gram samples of salicylamide were added to 40-cc. portions of artificial (14) pancreatic juice (*p*H 8.2), and digested as described above for both four- and eight-hour periods. Undissolved salicylamide was removed by suction, washed with sufficient cold water, air-dried, and weighed. The volume of filtrate was adjusted to 90 cc. Following acidification of the filtrate with 0.75 cc. of concentrated hydrochloric acid, the liberated salicylic acid was extracted with 5–35-cc. portions of ether, dried over Drierite, and evaporated to dryness.

Control for the above experiments was provided by extracting 0.150-Gm. samples of dissolved salicylamide with ether in the same manner as used in the experimental series. Each sample was contained in 40 cc. of artificial gastric juice to which was added 4.1 Gm. of $NaHCO_3$.

Results.—See Table I.

CONCLUSIONS

The results in Table I indicate that under experimental conditions designed to simulate those that might be encountered *in vivo*, the acid-amide linkage of salicylamide is resistant to cleavage. The results herein reported are in keeping with other reports in the literature for the hydrolysis of salicylamide (11), attesting to the stability of the compound in experiments designed to study *in vitro* behavior. Since there seems to be no evidence in the literature suggesting the presence of an amidase in the gastric contents, it is probably reasonable to conclude that salicylamide will pass into the small intestine unchanged.

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A Note on Antihistamines and Pentobarbital Anesthesia: Lack of Potentiating Effect*

By GEORG CRONHEIM and MICHAEL EHRLICH

THE most frequent side reaction of the majority of antihistaminics is the sedation and drowsiness caused by these drugs. This effect resembles the action of barbiturates, and it seemed of interest to determine whether certain antihistamines would increase the efficacy of pentobarbital. Such potentiating effect has been observed with a combination of barbiturates and methadone [Reutner and Gruhzit (1), and Cronheim and Ehrlich (2)]. The problem is also of practical importance inasmuch as it has been recommended to counteract the convulsive effect of an overdose of antihistamines by the administration of a sedative in the form of a short-acting barbiturate.

The drugs investigated were the hydrochlorides of the following N-N substituted N′-N′-dimethylethylenediamines:[1]

(*a*) 2-pyridyl-thenyl (methapyrilene hydrochloride)
(*b*) 2-pyridyl-5-chlorothenyl
(*c*) phenyl-thenyl
(*d*) phenyl-5-chlorothenyl

The compounds were given subcutaneously as a 10% solution in a dose of 10 mg./Kg. to a group of 10 fasting white rats with a weight of from 225 to 250 Gm. each, followed thirty minutes later by an

* Received April 27, 1951, from the Research Department of the S. E. Massengill Co., Bristol, Tenn.

[1] We are indebted to Monsanto Chemical Company for these compounds.

intraperitoneal injection of 30 mg./Kg. of pentobarbital sodium. Within one to three minutes after the second injection, the animals went into a state of complete flaccid prostration and the duration of the "sleeping time" was recorded. Each animal was allowed a rest period of one week between experiments.

Since it is known that the sleeping time after pentobarbital sodium may vary considerably and in an unpredictable manner, a control group of 10 rats was injected with pentobarbital sodium alone each time an experiment was made.

It has been found that pretreatment with the four antihistaminic compounds used in this study did not have any effect on the pentobarbital action. The average sleeping time varied from sixty to ninety-six minutes, as compared to sixty-three to eighty-three minutes for the five control series.

These results are in contrast to those reported by Winter (3). He found that using mice as test animals and Evipal or pentobarbital as the anesthetic agent, two antihistaminics of the ethylene diamine type (Pyribenzamine and Neo-Antergan) produced a slight increase of about 10% in the sleeping time of the animals. The effect of two other antihistaminics (Benadryl and R.P. 3277[1]) was much more pronounced.

It must be assumed that variations in the susceptibility of the two animal species,[2] and differences in the pharmacological properties of the antihistaminic compounds studied, are the reasons for the observed discrepancy.

SUMMARY

Attempts to potentiate the anesthetic action of pentobarbital with four antihistaminic compounds were unsuccessful.

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[1] R.P. 3277 is N-(β-dimethylamino-α-methylethyl)-phenothiazine, Societe des Usines Chimiques Rhone-Poulenc, Paris.
[2] The average sleeping time of mice after 50 mg./Kg. of pentobarbital was thirty-six minutes as compared to seventy-four minutes in rats after a dose of only 30 mg./Kg.

☆

Book Reviews

Mycotrophy in Plants. By ARTHUR P. KELLEY. The Chronica Botanica Co., Waltham, Mass.; Stechert-Hafner, Inc., New York City, 1950. xiv + 223 pp. Illustrated. 16 x 24 cm. Price $4.50.

Mycotrophy, in the literal sense of the word, means a response caused by fungi living in a kind of symbiosis on or in the roots of other plants. It developed as a science late during the past century when the classic writings of Albert Frank and Noel Bernard, who noticed this symbiosis among trees and orchids, began to interest botanists in the question, "Do these fungi actually exist for the assistance of cellular function among higher plants or are they essentially parasites which can be devoured by the host?" It is a difficult study because of the need to attach so much indirect evidence to the interpretation of any experimental results. In fact, even today little is known about the actual distribution in the plant kingdom of such structures called mycorrhizae. But, as is shown in Kelley's book, these symbiotic organisms appear to render a significant function in certain orchids, conifers, and other plants. The forester has long been interested in tree mycorrhizae for the economic importance attached to their role in tree seed germination. The student of plant nutriton and physiology finds some explanation however inconclusive, for nitrogen, carbohydrate, and fatty acid absorption through the activity of root mycorrhizae structures.

This text consists of 12 lectures, during which the author covers the subject by drawing heavily upon a large literature source. In addition to the treatment of mycorrhizae distribution, which is not meant to be entirely complete, the text includes interesting coverage of mycorrhizae structure with relation to root hairs and nodule formation. Biochemists will find Lectures XI and XII of particular interest. In these portions much attention is given to controversial theories such as the Romellian, Stahliam, and Bjorkman hypotheses of cellular absorption. These deal precisely with the role of mycorrhizae in inorganic and organic nutrition, particularly carbon and nitrogen up-take.

Although much experimental study needs to be completed before much of the function of mycorrhizae can be elucidated, the text presents the subject in an interesting fashion. It is a survey of the literature with some attempt to draw conclusions. In doing this, the author has used a very readable style. A biologist who finds himself rather indifferent at the beginning may really become quite speculative upon completing Kelley's 12 lectures. It is somewhat unfortunate, however, that more diagrams and plates were not included.—H. W. YOUNGKEN, JR.

Medicinal Chemistry. Vol. I. By ALFRED BURGER. Interscience Publishers, Inc., New York, 1951. xviii + 577 pp. 16 x 23.5 cm. Price $10.

Here is the first of two volumes of a treatise presenting an approach to medicinal chemistry for which individuals interested in that subject have recognized a need for a long time. The approach to the subject and its treatment by the author recognizes that a medicinal chemist must first of all be experienced in organic chemistry. In addition to this qualification, he needs to possess more than a superficial knowledge of biochemistry, pharmacology, microbiology, and other basic sciences contributing to the foundation materials of medicine. Professor Burger quite correctly stresses the fact that few individuals can be expert in more than one or two of these basic sciences. The medicinal chemist, to be successful, however, must be sufficiently well versed in the cognate sciences to visualize a composite picture of the many ramifications involved in the possible development of a synthetic organic compound from an idea into a drug.

In the actual evolution of a drug from an idea to clinical use, research teamwork among specialists in several different branches of the basic medical sciences is necessary. Obviously the better each member of a team understands the specialty of the other, the better it will function. Professor Burger's book can be used advantageously to accomplish a better understanding between chemists, pharmacologists, and clinicians.

For teaching purposes there has been a tendency in some pharmacy schools for instructors to classify organic medicinal chemicals according to structure. Some teachers of medicinal chemistry in pharmacy schools, on the other hand, have long maintained that a more rational approach is a classification according to pharmacological action and practical use. The proponents of this approach claim it is best, because of the lack of correlation between structure and physiological activity, and because the several activities exhibited by drugs in a single chemical classification make pharmacological discussions repetitive and a classification according to structure somewhat illogical. To support this contention, many examples can be cited comparable to that of the amines, which occur in analgetics, vasopressors, antihistamines, bacteriostatics, and antimalarials. Moreover, at least a year of organic chemistry and a course in pharmacology should constitute the minimum prerequisites for the study of medicinal chemistry, and this background makes a purely chemical classification of synthetic drugs unnecessary.

In this book, the author has arranged drugs according to their actual use. Drugs acting on various functions of the body are first considered. These functional or pharmacodynamic drugs will be followed in the second volume by drugs used in combating pathogenic microorganisms. A few special chapters, such as those on vitamins, parenteral fluid therapy, diagnostic agents (Volume I) and the hormones, chemotherapy and antibiotics planned for inclusion in Volume II are interspersed in suitable places.

The scope of Professor Burger's book is broad, but so is the subject. Excellent judgment has been exercised in the choice of subjects discussed and in the manner of presentation. Methods of biological testing are reviewed for each class of drugs, and the biochemical aspects of the subject are stressed throughout.

The reader is effectively oriented in relation to the subject in the first two chapters, which include an introduction and a brief history of the development of medicinal chemistry. Chapter III is devoted to a discussion of the relationship between chemical structure and biological activity, and is followed by a chapter on physical properties and their influence on biological activity. The principles and problems involved in the biological study of drugs are briefly but clearly discussed in the next chapter, while restricted responses of cells to drugs, including tachyphylaxis, habituation, tolerance, and acquired resistance are described in Chapter VI.

Following the excellent treatment of these background subjects, the author then proceeds to discuss individual classes of drugs, arranged according to their pharmacological activity. In general, the same design is followed for each group. Theories of action and certain fundamental biological and medical considerations are outlined and then the chemical aspects of well-chosen representative members of each group, usually now being used as drugs, are stressed. The discussion of the chemistry of synthetic drugs dispels any notion one might form from the content of the first six chapters that the book is a treatise on chemical pharmacology. The main emphasis throughout is on the chemical aspects of the subject.

The text is well documented with pertinent selected references, but no effort has been made to furnish complete bibliographies. This is as it should be in a textbook suitable for use by senior and graduate students. A short subject index to the first volume is provided, and a more comprehensive index for both volumes is planned for Volume II.

The aim of the author in writing this book is to familiarize the more advanced reader with past achievements of medicinal chemistry, with current thoughts and hypotheses, and with the direction of needed research in the whole field. This aim will, it is believed, be fully realized. The author and the publisher deserve a great deal of credit for making this book available at this time.

Despite the fact that the author considers *Medicinal Chemistry* a graduate treatise, this reviewer believes that it can be read and understood by senior students in most colleges of pharmacy in this country and can be used advantageously by such students either as a textbook or as a reference book.

Addendum 1951 to the British Pharmacopoeia 1948. Published under the direction of the General Medical Council. Constable and Co., Ltd., London, 1951. xviii + 114 pp. 14.5 x 23 cm. Price 17s. 6d.

A review of the *British Pharmacopoeia 1948* was published in the April, 1949, issue of the Scientific Edition of THIS JOURNAL, page 235. An addendum to this volume, official from September 1, 1951, has recently been published. In preparing the *Addendum*, the Commission has followed the general principles employed in the preparation of the *British Pharmacopoeia 1948*, which are outlined in the introduction to that volume. The *Addendum*

includes new monographs, and revisions of existing monographs, arranged alphabetically under the respective titles of those involved. Many additions and changes in the appendixes of the *British Pharmacopoeia* are also provided. Some of the changes in the *British Pharmacopoeia* made by this *Addendum* are in the field of antibiotics. The monographs on penicillin and dosage forms of penicillin have been revised and new monographs added on benzylpenicillin, procaine benzylpenicillin, three salts of streptomycin, dihydrostreptomycin, and chloramphenicol. The monographs on antitoxins, bacterial vaccines, and toxins have undergone quite extensive revisions and monographs have been added on a number of antihistaminic drugs, organic arsenical compounds, and sulfonamides. A group of preparations and materials derived from human blood has been included in a special section of the *Addendum*. Directions for the preparation of sterile solutions and suspensions for parenteral injections have been revised. Single-dose containers of injections must, according to new requirements, contain a specified volume in excess of the labeled volume. Procedures for the spectrophotometric method of assay for vitamin A are now recognized and new standard preparations for vitamin A and vitamin D are required in the assay outlined in the appendix. The British Pharmacopoeia Commission in preparing the *1951 Addendum* has maintained the high standard of performance exemplified in the *British Pharmacopoeia 1948*.

Everyone in the United States who has a copy of the latest *British Pharmacopoeia* will certainly want to acquire a copy of the *1951 Addendum*.

Methods of Vitamin Assay. 2nd ed. Prepared and edited by the Association of Vitamin Chemists, Inc. Interscience Publishers, Inc., New York, 1951. xviii + 301 pp. 16 x 23.7 cm. Price $5.50.

The second edition of this book, like the first, has been prepared by committees of the Association of Vitamin Chemists, Inc, with the cooperation of individual members and outside authors. The first edition, published in 1947, was well received and it is predicted that the second edition, with revised methods for vitamin assays and the inclusion of pertinent information from the current literature, will appeal strongly to biochemists, physicians, pharmacologists, and pharmaceutical chemists, particularly in the chemical, pharmaceutical, cosmetic, food, and related industries where vitamin analyses and assays are routinely or occasionally required.

In addition to having been completely revised, the book now includes assays for pantothenic acid, pyridoxine, folic acid, biotin, vitamin B_{12}, and niacin. The revision has been so extensive that the size of the book has been increased by more than one-half. The work is presented in 15 chapters, of which 12 relate to twelve individual vitamins, one to sampling, one to methods for determination of vitamins D, E, K, *p*-aminobenzoic acid, inositol, and choline, and a fifteenth chapter to the use of check samples in control of vitamin assay methods.

The book is well documented with many references at the end of each chapter and is provided with a thoroughly satisfactory index. The binding and printing are of good quality.

Optical Crystallography. 2nd ed. By ERNEST E. WAHLSTROM. John Wiley & Sons, Inc., New York, 1951. vii + 247 pp. Illustrated. 15.5 x 23.5 cm. Price $4.50.

This second edition of *Optical Crystallography* has been completely revised and expanded by approximately 50 pages. It reviews quite fully the principles of optical crystallographic theory. The immersion method of determining optical properties of crystals and fragments is particularly emphasized. The work definitely is not a handbook of the practical applications of crystallography. The author has apparently written the book with the idea in mind that once the student has firmly grasped the fundamental concepts of the subjects, he will then be able to cope with any problem in the field he is likely to encounter.

An outstanding feature of the book is a thorough explanation of the construction and manipulation of polarizing microscopes and accessories. Mathemathical derivations throughout the book have been limited, but sufficient have been included to supply information actually needed.

The new second edition of Professor Wahlstrom's book on optical crystallography includes an appendix containing a brief review of the universal stage method, many new illustrations, and a greatly expanded and modernized list of references.

The author, a consulting geologist and mineralogist as well as a teacher, has presented what appears to be sufficient basic information on crystallography to enable a student of the subject to acquire a sufficient understanding to apply it to practical problems such as those encountered in the analysis or identification of crystalline medicinal chemicals and other crystalline substances.

Active Carbon. By JOHN W. HASSLER. Chemical Publishing Co., Inc., Brooklyn, 1951. vi + 384 pp. 14.5 x 23.5 cm. Price $7.

This is a valuable book for industrial workers whose procedures require frequent use of active carbon, and for the investigator who wonders how he can utilize the bottle of active carbon on his shelf.

The author's interesting historical review cites the use of charcoal in medicine recorded in an Egyptian papyrus from 1550 B. C., then skips to the earliest recorded application of charcoal's adsorptive power for gases by the apothecary Scheele in 1773. Source materials for active charcoal and processes for its preparation are discussed. The bibliography at the end of this chapter includes many references to the patent literature. A bibliography is given at the end of each of the 34 chapters, and the book has a good index. The book is well written, with tabulations of important data where this is desirable. It appears that carbon with varying decolorizing power was used in the process for preparing the paper used in the printing of the book. There are large sections in which the pages vary from off-white to cream or buff colors. This in no way detracts from the value of the information the pages hold.

The chapter on biochemical preparations describes the role of active carbon in the preparation of penicillin, streptomycin, invertase, vitamin-bearing oils, vitamins A, C, D, G, and H, the antineuritic factor (thiamine) from yeast, folic acid from spinach, liver extract, the alcohol-ether precipitate factor from

liver, yeast, and milk, the *L. casei* factor from liver, insulin, oxytocic hormone, adrenal cortex hormone, plant wound hormone, and in the removal of pyrogens from parenteral solutions.

The book is divided into three sections as follows: Part I, Preparation and Properties; Part II, Applications; Part III, Experimental Methods. The author has moved along with new developments throughout his 35 years of work with active carbon, and he has the happy faculty of incorporating his own knowledge with the material he has accumulated from the literature.

This book should be available to all chemists and chemical engineers who might find the use of active carbon helpful if not essential in resolving many absorbing problems by adsorption phenomena.

Principles and Practice of Bacteriology. 4th ed. By ARTHUR H. BRYAN and CHARLES G. BRYAN. Barnes and Noble, Inc., New York, 1951. xv + 410 pp. Illustrated. 13.5 x 21 cm. Price $1.75.

Within the modest limits of its claim that it presents material for those "who are required to know the essentials of bacteriology," this book can be useful. It gives a superficial review of an enormous amount of information. It includes tabulated references of specific subject matter in 10 textbooks on bacteriology and a bibliography after each chapter.

It is surprising to note that only muzzling of dogs and the observation of incoming animals are given as the most efficient methods of prevention of rabies. Immunization of the entire dog population is under way in one county in the state in which one of the authors resides.

A useful glossary, in which one unexpectedly finds formulas for conversion of temperatures read on Fahrenheit or Centigrade thermometers, is included.

Perhaps the mose useful application of this book might be as an aid in a rapid survey of the field of bacteriology for the purpose of taking a general examination.

Examination questions of the National Board of Medical Examiners for the years 1946–1949; state board questions for nurses; and examination questions in veterinary bacteriology are given in the appendixes. The book has a good index.

Schimmel Briefs. Vol. I. Nos. 1–189, from April, 1935, to December, 1950. Schimmel and Co., Inc., New York, 1951. 195 pp. 22 x 28.3 cm.

The first issue of *Schimmel Briefs* was published in April, 1935. From that time on it has been the aim of Schimmel and Co., Inc., to use these briefs as a vehicle for furnishing information useful to the pharmaceutical, cosmetic, and soap manufacturers. Due to many requests from subscribers, the 189 issues appearing between April, 1935, and the end of December, 1950, have been reprinted and made available in a single volume. The earlier issues have been reproduced exactly as they originally appeared and in chronological order. It is especially interesting to compare some of the prices quoted in the issues appearing before 1940 with those of today. The value of this volume of *Schimmel Briefs* is greatly enhanced by an excellent index which makes it possible to locate information on any subject covered, in a very short period of time.

The publisher should be congratulated upon making this useful volume available to the pharmaceutical and allied manufacturers who use essential oils and aromatic chemicals in the fabrication of their products.

Accepted Dental Remedies. 16th ed. Council on Dental Therapeutics, American Dental Association, Chicago, 1951. xv + 207 pp. 16 x 20.5 cm. Price $1.50.

Accepted Dental Remedies is designed to serve as a handbook of dental therapeutics. In addition to functioning in this area, it also contains a list of therapeutic products marketed in the United States, which are acceptable to the Council on Dental Therapeutics.

Accepted Dental Remedies is published annually and this 16th edition conforms to the changes incorporated in the fourteenth revision of the *United States Pharmacopeia* and the ninth edition of the *National Formulary.* Among other innovations in the new edition is a modified classification of dental therapeutic products. The Council on Dental Therapeutics adopted a new procedure for a modified classification of dental products and only those classified in Group A (accepted) are listed.

The new procedure is completely described in the current edition of ADR. The book is provided with a useful list of more than 250 literature references, an index to distributors, and an unusually complete and well-designed general index.

Accepted Dental Remedies has improved with each annual edition and the current one is particularly outstanding.

Progress in Chromatography, 1938–1947. By L. Zechmeister. John Wiley and Sons, Inc., New York, 1951. xviii + 368 pp. Illustrated. 14.5 x 22.5 cm. Price $8.

The work in this rapidly expanding field that was reported during the years from 1938 to 1947 is reviewed. The critical appraisal of the reported results and claims is concise and interesting. This is especially so when fundamental theories and ideas are offered. At times, however, the reviews are too brief. Professor Zechmeister indicates clearly his belief that most of the experimental data in this field will be obtained by methods of trial and error, although in some cases, under certain conditions, predictable results can be obtained. He cites DeVault's equations which predict that for a single solute whose adsorption isotherm is of the normal type, upon development, a sharp bottom boundary and a gradual tailing in the top section of the broadening zone will appear. On the other hand, in the separation of *cis-trans* isomers, it cannot be postulated that either isomer will be more strongly adsorbed. The influences of polar solvents and types of adsorbents in altering relative orders of separations are discussed.

The book is written in abstract style and is valuable as a reference volume because it is well indexed and contains an extensive bibliography. The extent of the author's contributions in the field of chromatography during the period reviewed is attested

by the numerous references to his works. An interesting discussion is given on the fact that some polarizing adsorptions are irreversible in the sense that upon elution with a polar solvent a chemically changed substance is recovered. The lack of a large number of reliably standardized commercial adsorbents of very different composition and grade is cited as one of the main factors preventing the more rapid progress of chromatography. Methods for the standardization of adsorbents are also described.

The field of chromatography has shown rapid progress since 1947. The book contains very few references to paper chromatography, and the sparse treatment of this phase of development indicates the already existing need for a review of the papers on the applications of chromatography that have appeared after the period reviewed in this book.

History of Pharmacy. 2nd ed. By EDWARD KREMERS and GEORGE URDANG. J. B. Lippincott Company, Philadelphia, 1951. xiv + 622 pp. Illustrated. 15.5 x 23.5 cm. Price $7.50.

Since the publication of the first edition of the Kremers-Urdang *History of Pharmacy* in 1940, new historical material of especial pharmaceutical interest has evolved. The purpose of the new edition at this time is to present new findings that have resulted from research and to record some pharmaceutical developments of the past ten years. The arrangement, whereby a sociologic integration instead of a chronologic narration of the historical details was so effectively accomplished in the first edition has been maintained in the second.

According to the preface, "the new book has been changed in content but not in its spirit and its goal. It has remained 'a guide and a survey,' to be used for teaching as well as for reading, as a book of reference as far as data and dates are concerned, and a book presenting and evaluating concepts."

Among the several innovations of the new edition is a chapter on the development of pharmacy in Spain contributed by Professors Guillermo Folch Jou and Rafael Folch Andreu of the School of Pharmacy of the University of Madrid.

The American Institute of the History of Pharmacy and the Kremers-Urdang *History of Pharmacy* have done much to stimulate interest in the United States in pharmaceutical history. The appearance of a new edition now will undoubtedly contribute to a continued interest in the subject and will be instrumental in stimulating an interest in history among pharmacy students and imparting to them knowledge of the rise and development of their profession.

Pharmaceutical Botany. 7th ed. By HEBER W. YOUNGKEN. The Blakiston Company, Philadelphia, 1951. xviii + 752 pp. Illustrated. 16 x 23.5 cm. Price $7.

This book is well known to several generations of pharmacists who used it during student days as a textbook, and later as a reference. The excellence of Professor Youngken's *Pharmaceutical Botany* is attested by the fact that a seventh edition has been published.

Anyone will be convinced, by a perusal of the new edition, that botany is a dynamic science. Professor Youngken has included new material on plant metabolism and plant environment as well as new information relating to anatomy. Plant hormones, chemical growth regulators, and vitamins are adequately discussed.

Additional material on general basic botanical principles makes the book suitable for a course in general botany as well as pharmaceutical botany. The material is skillfully arranged to lead the student gradually from the simpler and less technical to the more advanced aspects of the subject.

The chapter on classification and naming of plants is completely up to date with modern usage. Effective teaching aids include a glossary, list of reference works, and an unusually complete and easy-to-use index.

BOOKS RECEIVED

Industrial Oil and Fat Products. 2nd ed. By ALTON E. BAILEY. Interscience Publishers, Inc., New York, 1951. xxiv + 967 pp. Illustrated. 16 x 23.5 cm. Price $15.

Substances Naturelles de Synthese. Vol. I. By LEON VELLUZ. Masson & Cie, Paris, 1951. 141 pp. 16 x 23.3 cm.

Unification of Pharmacopoeias. World Health Organization Technical Report Series No. 35. Columbia University Press, International Documents Service, 2960 Broadway, New York, 1951. 32 pp. 16 x 24 cm. Price $0.20.

Preparation of Organic Intermediates. By DAVID ALLEN SHIRLEY. John Wiley and Sons, Inc., New York, 1951. x + 328 pp. 15.5 x 23.5 cm. Price $6.

The Vitamin B Complex. By F. A. ROBINSON. John Wiley and Sons, Inc., New York, 1951. xi + 688 pp. 14.5 x 22.5 cm. Price $9.

An Introduction to Organic Chemistry. 7th ed. By ALEXANDER LOWY, BENJAMIN HARROW, and PERCY M. APPELBAUM. John Wiley and Sons, Inc., New York, 1951. xiv + 480 pp. Illustrated. 14.5 x 22 cm. Price $5.

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Whether your laboratory is large or small the Beckman Automatic Titrator provides important advantages in your titrating operations . . .

- *It releases the technician during titration, enabling him to perform other operations such as preparing samples, or calculating results.*
- *It eliminates the fatigue caused by close observation required in manual procedures.*
- *It gives objective, reproducible results . . . eliminates errors due to personal factors.*
- *It provides time-saving conveniences for sample handling.*

PERFORMS A WIDE VARIETY OF TITRATIONS—Neutralization, Oxidation-Reduction, Precipitation, Complex-Formation and other types.

NO SPECIAL TRAINING IS REQUIRED—rapid and accurate titrations can readily be performed without special skill or technique.

QUICK, SIMPLE OPERATION—completes many routine titrations in only 1-1½ minutes—even titrations to 0.1% accuracy in 2½ minutes or less. Change of sample is simple, rapid—a single motion raises, locates and secures new sample in operating position.

CONVENIENT, VERSATILE, ADAPTABLE—0° to 100° C temperature compensation . . . adjustable holder accommodates 10 ml to 400 ml beakers or similar vessels . . . instrument may be used with all standard burettes down to 5 ml . . . as many as four delivery units accommodated by single amplifier control units . . . uses standard Beckman electrodes . . . electrode holders and delivery tip can be pivoted into any required position . . . ample provision for mounting heating devices or other special equipment.

ALSO A RELIABLE pH METER—the Beckman Automatic Titrator can also be used as an AC-powered pH meter to give accurate readings over the range 0 to 14 pH, as well as millivolt readings from −600 to +1400 mv.

For full details on this new Beckman instrument see your authorized Beckman dealer—or write direct. *Beckman Instruments, National Technical Laboratories, South Pasadena 42, California.*

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